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COMPLEX SEGREGATION ANALYSIS OF DENTAL DECAY IN AN ISOLATED POPULATION FROM NORTH OF BRAZIL

RENATA IANI WERNECK

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2010

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“**Dedico minha tese de doutorado aos meus pais, à minha irmã e à minha sobrinha/afilhada.**”

“I dedicate my PhD thesis to my father and mother, my sister and my niece/goddaughter.”

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"…Et il revint vers le renard:

- Adieu, dit-il...

- Adieu, dit le renard. Voici mon secret. Il est très simple: on ne voit bien qu'avec le cœur. L'essentiel est invisible pour les yeux.

- L'essentiel est invisible pour les yeux, répéta le petit prince, afin de se souvenir.

- C'est le temps que tu as perdu pour ta rose qui fait ta rose si importante.

- C'est le temps que j'ai perdu pour ma rose... fit le petit prince, afin de se souvenir.

- Les hommes ont oublié cette vérité, dit le renard. Mais tu ne dois pas l'oublier. Tu deviens responsable pour toujours de ce que tu as apprivoisé. Tu es responsable de ta rose...

- Je suis responsable de ma rose... répéta le petit prince, afin de se souvenir."

"...E voltou, então, à raposa:

- Adeus, disse ele...

- Adeus, disse a raposa. Eis o meu segredo. É muito simples: só se vê bem com o coração. O essencial é invisível para os olhos.

- O essencial é invisível para os olhos, repetiu o principezinho, a fim de se lembrar.

- Foi o tempo que perdeste com tua rosa que fez tua rosa tão importante.

- Foi o tempo que eu perdi com a minha rosa... repetiu o principezinho, a fim de se lembrar.

- Os homens esqueceram essa verdade, disse a raposa. Mas tu não a deves esquecer. Tu te tornas eternamente responsável por aquilo que cativas. Tu és responsável pela rosa...

- Eu sou responsável pela minha rosa... repetiu o principezinho, a fim de se lembrar."

(Antoine de Saint-Exupéry, "***Le Petit Prince***", Gallimard, 1946. p. 76, cap XXI)

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ABSTRACT

Dental decay is a chronic, complex disease and one of the most common diseases in the population. It is widely accepted that the occurrence of dental decay depends on environmental and host-related factors, such as diet, biofilm composition, host susceptibility and time of exposure. Over the last years, studies using experimental animal models, as well as human observational (familial aggregation and twin studies), linkage and association analysis were conducted aiming to demonstrate the existence of genetic factors influencing dental decay. However, due to the complex characteristic of the trait, questions such as the model of inheritance and parameters as the frequency and penetrance of the deleterious allele are yet to be addressed. We conducted a Complex Segregation Analysis (CSA) using as phenotype both the quantitative Decayed, Missing and Filled Teeth index (DMFT) and the number of Decayed Teeth (DT) in a sample of extended multiplex families from an isolated population from Amazonic state of Pará, north of Brazil. A major gene effect controlling resistance to both phenotypes was detected, with the best-fit model being co-dominant for DMFT and dominant for DT. For DT, the frequency of the resistance allele *A* was 0.63 and mean DT was 1.53 and 9.53 for genotypes AA/AB and BB respectively. Our data indicates that the genetic model detected for DMFT is likely the result of a combination of independent genetic mechanisms controlling the components of the index. The CSA is the first step towards a comprehensive description of the exact nature of the genetic risk factors controlling human susceptibility to dental decay. A deeper understanding of the genetic aspects of dental decay pathogenesis will ultimately lead to new strategies for prevention of such prevalent disease worldwide.

Key-words: caries - genetics - genetic epidemiology - susceptibility - inheritance

RESUMO

A cárie dentária é uma doença crônica, complexa e uma das enfermidades mais comuns na Odontologia. É amplamente aceito que a ocorrência da cárie depende de fatores ambientais e fatores relacionados com o hospedeiro, como dieta, susceptibilidade do indivíduo, composição do biofilme e tempo de exposição. Ao longo dos últimos anos, estudos experimentais com modelos animais, como também estudos observacionais humanos (agregação familial e estudos em gêmeos), análises de ligação e associação vem sendo conduzidas com o intuito de demonstrar a existência de fatores genéticos influenciando a cárie. Entretanto, devido à natureza complexa da doença, questões relacionadas com o modelo de herança genético e parâmetros como frequência e penetrância do alelo deletério ainda não foram discutidas. Nós conduzimos uma Análise de Segregação Complexa (ASC) utilizando dois fenótipos quantitativos: índice de Dentes Cariados, Perdidos e Obturados (CPOD) e número de Dentes Cariados (CD) em uma amostra populacional de famílias multiplex estendidas residentes em uma Colônia isolada na Amazônia, estado do Pará, norte do Brasil. Um efeito genético principal controlando resistência para ambos os fenótipos foi detectado, sendo que para o fenótipo CPOD o melhor modelo foi codominante e para o fenótipo CD, dominante. Para o fenótipo CD, a frequência do alelo de resistência *A* foi 0,63 e a média do fenótipo CD foi de 1,53 e 9,53 para os genótipos AA/AB e BB, respectivamente. Nossos resultados indicam que o modelo detectado para CPOD é provavelmente o resultado da combinação de mecanismos genéticos independentes controlando os componentes do índice. A ASC é o primeiro passo para a melhor descrição e compreensão da exata natureza dos fatores de risco genéticos controlando a susceptibilidade humana à doença cárie. Uma melhor compreensão dos aspectos genéticos envolvidos na patogênese da cárie poderá originar novas estratégias de prevenção para esta doença com alta prevalência no mundo.

Palavras-chave: cárie – genética – genética epidemiológica – susceptibilidade – herança genética

1. INTRODUCTION AND LITERATURE REVIEW

1.1 DENTAL DECAY

1.1.1 Dental Decay: Definitions

Dental caries is a complex, chronic, multifactorial disease (Fejerskov, 2004; Departament of Health and Human Services, 2005) and one of the most common diseases in Dentistry, together with periodontal disease and malocclusion (Fejerskov, 2004; Brathall and Hänsel, 2005). The World Health Organization defines dental caries as a “localized, post-eruptive, pathological process of external origin involving softening of the hard tissue and proceeding to the formation of a cavity” (WHO, 2004). Dental decay has an important role in the development of tooth loss and dental pain, which have been associated with problems in school and absenteeism at work (Fejerskov, 2004; World Health Organization, 2004), leading to a decrease in quality of life (Petersen, 2003). Moreover, oral health presents close association with the individual general health and may be a risk factor for several diseases (Petersen, 2003). Finally, traditional treatment of this oral disease is costly in industrialized and low-income countries (Petersen, 2003).

Dental decay can be seen as a series of events organized in a time frame: the disease starts with mineral loss through the initial enamel or root lesion, progresses to dentinal involvement and culminates with eventual cavitation. The mineral loss happens due to the disequilibrium between two events in the enamel: demineralization and remineralization (Fejerskov and Manji, 1990), a continuous, dynamic process that determines the end result (Feathrstone, 2004). The disease is characterized in the first days by a whitish and opaque lesion. The enamel on the lesion is more porous, because the mineral removed is from the internal part of the tissue. The cavities are just the consequence of the disease.

1.1.2 Epidemiology

In the early XX century, two measurement systems to estimate dental decay were proposed: the proportion of first molars lost through decay (Hyatt, 1920) and the percentage of permanent teeth affected (Ainsworth, 1920). Both of these methods were useful when there was little information about the disease, but they lack in sensitivity. In 1937, Klein and Palmer were requested to conduct an epidemiological survey with native children from the entire United States territory; as one of the consequences, the “DMF” (decay, missing and filled teeth or surface) index was created (Klein and Palmer, 1937) to be used for permanent (DMFT) and deciduous (dmft) teeth, as well as for teeth surface (DMFS). The idea of the DMF index was to change dental decay evaluation from the individual level to the teeth level, by counting and describing affected teeth (Klein and Palmer, 1938; Klein and Palmer, 1940). Moreover, the authors proposed to use the DMF not only as a measurement of disease prevalence but also, severity. Over time and until today, the DMF index became the most common and widely accepted measurement for dental decay in the general population. The major advantage of the DMFT index is that, because of its widespread use worldwide over the past 60 years, it provides a reasonably accurate historical account of changes in the prevalence of dental caries.

The DMFT has been generally decreasing in the last few years in developed and developing countries. According to the World Health Organization (WHO) (World Health Organization, 2004), the DMFT index in children aged 12 years old was higher than 3.0 in 49% of the 184 reporting countries in 1980. In 2000, this value for children at the same age was equal or lower than 3.0 in 68% of the same countries studied (World Health Organization, 2004), showing a decline of the disease in the last 20 years, even in developing countries (Peterson, 2005). Such information seems satisfactory for health professionals; however, WHO declares: *“There is a perception that dental decay is not anymore a problem in developed countries, but, this disease still affects from 60 to 90% of children at school age and the majority of adults. Also, dental decay is the mouth disease most prevalent in countries of Asia and Latin America”* (World Health Organization, 2004).

In Brazil, according to data collected in the last epidemiological surveillance conducted in 2003 and including all regions of the country, mean DMFT was 2.78, 6.17, 20.1 and 27.79 in individuals from age classes up to 12, 15-19, 35-44 and 65-74 years old, respectively, with the decayed teeth component of the DMFT causing the major impact (Costa, Solla *et al.*, 2003). This emphasis in the decayed component shows that e ven though the Brazilian mean is almost equal to the global mean, dental decay still has a major impact in the Brazilian Public Health system.

Numerous studies have shown that the global decline in dental caries prevalence is non-homogeneous across countries (Bonecker and Cleaton-Jones, 2003; World Health Organization, 2004). Even though strategies, such as the use of topical fluoride, sealant and diet control have been developed and used by dental professionals to prevent dental decay, their efficiency to eliminate the disease is not clear. (Petersen, 2003; Dye, Tan *et al.*, 2007). Dental caries, early dental loss and edentulism seem to concentrate in some groups of individuals. This phenomenum, termed *polarization* has been exhaustively discussed, but its causes remain obscure (Pine, 2005; Narvai, Frazão *et al.*, 2006).

1.1.3 Predictive Factors: Determinants and Modifiers

Since the 1960s, dental decay development has been suggested as the result of interaction between four major factors: biofilm, diet, time and host (Keyes, 1960; Keyes, 1962; Evans, LO *et al.*, 1993). Moreover, for the past four decades, different authors have described gender, ethnicity and age as additional risk factors for the disease progress (Evans, LO *et al.*, 1993; Antunes and Peres, 2006).

When biofilm is exposed to highly fermentable carbohydrates, cariogenic bacteria are selected, modifying the biofilm composition (Cury, Rebelo *et al.*, 2000; Nobre dos Santos, Melo dos Santos *et al.*, 2002). The cavities occur where the biofilm persist during long periods. The cavity will be an ecological niche where the microorganisms will persist and adapt to the reduced pH. If the biofilm persists, the cavitation will increase (Fejerskov, 2004; Kidd, 2004). The cavities happen in

sites where the bacteria develop a biofilm if the latter are not removed or disorganized by mechanical forces, such as friction, abrasive toothbrushing and use of floss (Fejerskov and Kidd, 2005).

The main reported cariogenic bacteria are *Streptoccocus mutans (S. mutans), S. sobrinus* and some species of *Lactobacillus* (Keyes, 1962; Feathrstone, 2004). Bacterial growth will cause a decrease of the pH of the plaque, increasing the cariogenic potential (van Houte, 1994). Continuous exposure to acids produced by these bacteria could lead to dental decalcification (Feathrstone, 2004). Thus, when a more cariogenic biofilm occurs and the buffering capacity of the host cannot compensate the acid attack, dental cavities may appear. Also, salivary flow and saliva composition are important to the biofilm etiopathogenicity (Lenander-Lumikari and Loimaranta, 2000). The major role played by saliva is in the regulation of the exposure of tooth surfaces to carbohydrate and plaque acidity, hence, the microbial composition and cariogenic potential of dental plaque (van Houte, 1994).

Environmental factors, such as behavioral habits (Fejerskov, 2004), may also influence the development of dental decay. Socioeconomical status (SES) is a non-biological determinant factor often related to educational level, the perception of the individual about his/her own health, life style, dietary composition and access to dental care (Antunes and Peres, 2006; Bastos, Gigante *et al.*, 2007), being all these examples of environmental risk factors. Lower SES is associated with financial, social, and material disadvantages that compromise one's ability to care for himself/herself, to seek and obtain professional health care and to live in healthy environments. In most countries, the socioeconomic gradient in use of dental services is well documented, not only in terms of relatively lower frequency of dental visits for low-income and less-educated individuals, but also in relation to lower access of preventive services (Watt and Sheiham, 1999). Family size is also a risk factor for dental decay: individuals from large families have more probability to show higher DMFT index (Evans, LO *et al.*, 1993).

Hygiene habits are also correlated with educational level and SES (Adair, Pine *et al.*, 2004) and the frequency of tooth brushing has been shown to influence the amount of caries (Chesters, Huntington *et al.*, 1992; Kriger, 1999). However, this is not a consensus: studies have failed to prove that brushing has a

clear association with caries incidence. A possible explanation may be the existence of recall and social desirability response bias (Aday, 1996; Livny, Vered *et al.*, 2008; Martins, Ramos-Jorge *et al.*, 2008). Also related to hygiene habits, the use of fluoride toothpaste is an important method of delivering fluoride to the tooth surface, which will enable a quickly remineralization (National Institute of Health Consensus development Conference Statement, 2001). Access to fluoridated water is also a variable contributing to the decline of decayed teeth (Krasse, 1996; Antunes and Peres, 2006; Griffin, Regnier *et al.*, 2007).

Modifying factors specific to the tooth are important to consider. The level of enamel mineralization, which will increase or decrease the resistance to acid dissolution is a factor influencing the teeth susceptibility to caries. This level of mineralization is influenced by intrinsic (during the tooth formation) and extrinsic factors (environmental and local factors) (Lima, 2007). Also the tooth, as well as pit and fissures morphology can influence tooth susceptibility to dental decay (Grainger, Paynter *et al.*, 1959; Grainger, Paynter *et al.*, 1966).

However, the combination of all aspects mentioned above does not entirely explain disease outcome. Individuals exposed to the same levels of environmental factors present differences in the DMFT index (Pine, 2005). Those differences may suggest the influence of genetic factors in the etiopathogenesis of dental caries.

1.1.4 Clinical Manifestation and Diagnosis

The earliest changes to dental enamel are subclinical: subsurface demineralization including inaccessible sites. This event is not easily observed and can be identified in extracted teeth submitted to histological exam. The next stage is characterized by the development of enamel lesions, usually with apparently intact surfaces. These non-cavitated enamel lesions are already a stage of dental caries and not a pre-disease state (Kidd and Beighton, 1996). Finally, there is the development of visible enamel, dentine and pulpal caries, identified because they are extensive and more clinically obvious lesions (Pitts, 2004).

The initial caries lesion in the enamel is characterized by a whitish color, opaque and porous. This initial lesion can be observed when the tooth is dried. With the evolution of the lesion, it is not necessary anymore to dry the lesion to identify it. The initial lesion in the root is characterized by white or yellow spots in the enamel of the root surface. When the lesion is active it is white, porous and opaque in the enamel and the dentin tissue is soften and presents a light brown color. Inactive lesions are white but bright or pigmented and smooth in the enamel, and the dentin tissue is dark and hard (Kriger, 1999).

Different methods have been used to diagnose dental decay. The main methods are: visual or visual-tactile and radiographic exams, translumination with optical fiber and electric resistance test (Fejerskov, 2004; Departament of Health and Human Services, 2005). None of them is considered completely efficient and effective (Pereira, 1997; Kriger, 1999). Diagnosis of caries follows two steps: detection of a lesion (implies an objective method of determining whether or not disease is present) and lesion assessment (aims to characterize or monitor a lesion, once it has been detected) (Fejerskov and Kidd, 2005).

As cited above, dental decay is a cavity that happens as consequence of a disequilibrium of the demineralization-remineralization process. In the past, diagnosis was conducted to detect the lesion caused by this disequilibrium. Nowadays, dental decay is known as a multifactorial disease and the focus is no longer the detection of the lesion, but also to comprehend the patient and all the factors that could be influencing the disease development (Lima, 2007).

1.1.5 Prevention and Treatment

Treatment can be non-invasive (or preventive) and invasive. Dental decay is chronic, slow progressive and one of the most common preventable diseases (Fejerskov, 2004). It is reversible in the initial phase and generally can be interrupted at any time, even when the enamel and the dentin are already destroyed (Feathrstone, 2004).

The mechanical control of the biofilm allows remineralization mediated by saliva, keeping the enamel lesion in a reversible stage during a certain period of time (Lenander-Lumikari and Loimaranta, 2000). If this control is periodic, then the demineralization-remineralization process can be rebalanced and the lesion can be paralyzed.

Studies have shown that diet control can help decrease caries development (Nobre dos Santos, Melo dos Santos *et al.*, 2002). The main strategy is to control the frequency of the consumption of cariogenic diet, mainly containing sucrose. According to Burt and Pai, in a review in 2001, controlling the consumption of sugar remains a justified part of caries prevention; however, not always the most important aspect (Burt and Pai, 2001).

The water fluoridation has been observed as a preventive factor for dental decay, helping decrease caries around 15% (Klein, 1946; Bruce and Gunter, 1953; Jones, 1997; Yeung, 2007). This initiative is regulated by the WHO as well as by the Brazilian Ministry of Health. The use of toothpaste containing fluoride has been discussed for more than 50 years and has been described as an important factor to decrease dental decay in around 25%, according to different studies (Cury, Tenuta *et al.*, 2004; Secretaria Municipal de Saúde de Curitiba - Centro de Informação em Saúde, 2006). Solutions containing sodium fluoride (0.05% or 0.2%) has also been prescribed as a preventive tool, together with the use of fluoride gel (9000 to 12300 ppm F) and varnish fluoridate (22600 ppm F) by dental care professionals (Secretaria Municipal de Saúde de Curitiba - Centro de Informação em Saúde, 2006).

Another preventive treatment can be conducted using antimicrobials; however, with inconclusive evidence for caries prevention in risk groups (Twetman, 2004) perhaps due to the unique organization and adherence of the microorganisms in the biofilm (Fejerskov and Kidd, 2005). Furthermore, different from other infectious diseases, dental decay is caused by oral resident microorganisms with a potential beneficial function on a healthy mouth. Consequently, the goal should not be to eliminate them, but to control them in a compatible health level. The chlorhexidine is the most used anti-plaque agent. It is absorbed by the mucosa and teeth surface and it is retained during a period of time

that assures a longer and constant exposure of the plaque to the antimicrobial agent (Fejerskov and Kidd, 2005). The chlorhexidine effect is better observed when applied by a professional combined with a rigorous prophylactic regime involving oral hygiene, dietary control and professional prophylaxis (Zickert, CG *et al.*, 1982).

When the lesions are active, the treatment is simply based on the mechanical removal of the lesions and also helping the individual to control plaque. However, in a more modern approach, many dentists no longer take a narrow surgical view seeking to apply intervention treatment as a one-off event at a certain trigger point of disease severity. The evidence that caries is an initially reversible, chronic disease with known multifactorial etiology is being increasingly appreciated. The caries process should be followed over time by individualized but also community-based preventive treatment. Preventive procedures can be conducted before the initial cavities start, leading to appropriate treatments for each stage of the disease. Furthermore, the process needs to be monitored by both dentists and patients in a long-term, even lifetime initiative (Fejerskov and Kidd, 2005).

1.2 HUMAN GENETICS OF COMPLEX DISEASES

Human genetic diseases are classified into two categories: (i) Mendelian diseases, and (ii) complex diseases. Mendelian diseases are rare, usually caused by variations in one single gene (monogenic) and presenting a perfect correlation between genotype and phenotype. Complex diseases are the result of the interaction between genetic (often polygenic) and non-genetic factors (Strachan and Read, 2002; Sørensen, Nielsen *et al.*, 1988). Dissecting the genetic component of a complex disease is not a trivial task; however, understanding the genetic basis of susceptibility to these frequent diseases will produce a deep impact over public health systems, as well as over the lives of millions of individuals worldwide. Examples of complex diseases with a known genetic component are infectious disorders such as leprosy (Mira, Alcaïs *et al.*, 2003; Mira, Alcaïs *et al.*, 2004; Ranque, Alcais *et al.*, 2005; Mira, 2006; Moraes, Cardoso *et al.*, 2006; Alcais, Alter *et al.*, 2007; Ranque, Alter *et al.*, 2007), tuberculosis (Fieschi, Dupuis *et al.*, 2003; Remus, El Baghdadi *et al.*, 2004; Baghdadi, Orlova *et al.*, 2006), and oral illnesses such as periodontitis (de Brito Junior, Scarel-Caminaga *et al.*, 2004; Souza, Trevilatto *et al.*,

2005) and dental decay (Finn and Caldwell, 1963; Beck and Drake, 1975; Boraas, Messer *et al.*, 1988; Conry, Messer *et al.*, 1993; Bonecker and Cleaton-Jones, 2003).

Several definitions of genetic epidemiology are available in the literature. Morton and Chung, in 1978, defined genetic epidemiology as "*the study of the etiology of disease among groups of relatives to unravel the causes of family resemblance and study the inherited causes of disease in populations*" (Morton and Chung, 1978). Other authors emphasize the role of genetic epidemiology in studying genetic-environmental interactions in disease etiology (Cohen, 1980; Philippe, 1982). In summary, genetic epidemiology may be defined as the study of the joint action of genes and environmental factors causing disease in human populations (Morton, 1982; Neel, 1984; Rao, 1985; Last, 1993). The broad goal of genetic epidemiology is to understand the role of genetic factors controlling disease in human populations with the ultimate objective of disease control and prevention (Khoury, Beaty *et al.*, 1993).

1.2.1 Observational Genetic Epidemiology: Genetics without DNA

An early stage of genetic epidemiology studies involves the description of a genetic component controlling the phenotype of interest and the parameters of the correspondent genetic model. In these studies, essential information – but not biological samples, such as DNA – is used to contextualize epidemiological, SES, clinical and demographical data into pedigrees. The concept may be adapted to animal models, typically using inbred strains presenting extreme known phenotypes to analyze the result of controlled crossing, correlating genotypes and phenotypes (Kanamoto, Nonaka *et al.*, 1994). Since controlled crossings are not applicable to human populations, an alternative, exclusively observational analysis is necessary, such as familial aggregation, twin studies and complex segregation analysis (Figure - 1).

1.2.1.1 Familial Aggregation

Familial aggregation analysis investigates the clustering of disease cases in large pedigrees that can be caused by excess sharing of genetic variants, environmental variants or both (Burton, Tobin *et al.*, 2005). The exposure to environmental variables is often assumed equal between members of the same family. When conducting a genetic analysis, no attempt is made to determine the cause of aggregation; yet, the objective is solely to observe clusterization of cases. The aggregation is often measured as correlation or λ_R (the risk of disease for a consanguineous person of the proband as compared to the general population). Importantly, familial aggregation analysis has no power to distinguish whether clusterization is caused by sharing of biological or environmental factors (Strachan and Read, 2002).

1.2.1.2 Twin Studies

The objective of twin studies is to distinguish between genetic and non-genetic factors contribution to familial aggregation of a phenotype. The rationale is straightforward: monozygotic twins (MZ) share 100% of their alleles, whereas dizygotic twins (DZ) share 50% of their alleles just as any non-twin sibpairs. One can consider most differences between MZ being due to non-genetic influence; in DZ twins, differences will be of mixed nature (genetic and environmental). The aim of twin studies is to compare intra-class correlation between MZ and DZ twin pairs and/or calculate the heritability (the proportion of the phenotypic variability due to genetic variance) of the trait. Importantly, twins normally share the same intra-uterus and early post-natal conditions; therefore, any environmental influences can be minimized (Shuler, 2001; Brathall and Hänsel, 2005; Townsend, Hughes *et al.*, 2008). A sophistication of the design is introduced when twins reared apart are studied, providing even more powerful results (Boraas, Messer *et al.*, 1988; Conry, Messer *et al.*, 1993).

1.2.1.3 Complex Segregation Analysis

To obtain genetic parameters not estimated by familial aggregation and twin studies, Complex Segregation Analysis (CSA) can be applied. This is a complex statistic method, which can be viewed as a sophistication of familial aggregation analysis, now focusing on the pattern of aggregation within individual families (Hassel, 1995; Burton, Tobin *et al.*, 2005) and generally carried out before and to justify expensive molecular studies. In CSA, the aim is to find a major gene effect, taking into account both genetic and environmental factors influencing the phenotype. Importantly, the term “major gene” means its effect is strong enough to be distinguished from other gene effects, but does not assume it is the only gene involved. Elston and Stewart (Elston and Stewart, 1971) define CSA as “*the statistical methodology used to determine from family data the mode of inheritance of a particular phenotype, especially with a view to elucidating gene effects*”. In a CSA, information about the pedigrees and phenotypic data is compared using the maximum likelihood test. The analysis includes genetic models of inheritance (dominant/co-dominant/recessive), allelic frequencies and penetrances, aiming to obtain the best likelihood across all variables and tested models (Thomas, 2004; Burton, Tobin *et al.*, 2005). The parameters of the genetic model defined by a CSA may be of great use in subsequent, genetic mapping studies, as discussed below. However, CSA has one major limitation: the method does not produce information about the exact genetic nature of all genes and sequence variations involved.

One important issue to be addressed in CSA is the risk of ascertainment bias. In human genetic analysis data are collected usually following a procedure in which the proband is ascertained first, initiating a sequential sampling scheme (Tai and Hsiao, 2001). However, this sampling method is non-random and, in most of the cases, causes biased estimation which may be intractable. An extensive review on CSA, which is the focus of this study, will be given below (in section 1.2.3)

1.2.2 Molecular Genetic Epidemiology: Genetics with DNA

Once evidence of a possible genetic component influencing complex disease is obtained (without genotyping), the next step is to locate and identify any causative regions and genes. Molecular genetic epidemiology tools can then be applied to advance in the understanding of the disease by searching for modifications in the DNA (mutations and polymorphisms used as genetic markers) that correlates with the phenotype, with or without an apparent biological function. Molecular genetic epidemiology strategies involve linkage and/or association analysis (Figure – 1).

1.2.2.1 Linkage Analysis

Linkage analysis is a family-based study aiming to find chromosome regions containing genes related to the disease (Dawn Teare and Barrett, 2005). The goal of this analysis is to find non-random segregation of chromosomal loci and phenotypes of the disease being mapped. If genetic and disease markers co-segregate, regions containing candidate genes for the study disease are identified.

Linkage analysis can be parametric (model-based) and non-parametric (model-free). In parametric analysis, it is necessary to specify parameters of the genetic model involved as obtained from CSA (Burton, Tobin *et al.*, 2005). The main objective is to estimate the recombination fraction, estimated as the frequency of recombinants generated during meiosis (Strachan and Read, 1999), between individual markers of known location and the disease locus (Teare and Barrett, 2005). The idea is that the closest two loci (for example, the disease and the molecular marker locus) are on the chromosome, the less likely is that they will be separated by recombination during meiosis – therefore, recombination fraction will tend to zero (Thomas, 2004). Alternatively, non-parametric linkage analysis is conducted when no genetic model was inferred by CSA. This model-free analysis is usually conducted using sibling pairs, but can also be done using other groups of relatives. The rationale is that, between affected relatives, excess sharing of haplotypes that are identical by descent (IBD) in the region of a disease-causing

gene would be expected, irrespective of the mode of inheritance (Teare and Barrett, 2005).

Linkage analysis has been classically used to map genes related to specific phenotypes and, over the past decade, to locate disease genes based exclusively on their gene position, a strategy known as Positional Cloning.

1.2.2.2 Association Analysis

Association analysis (family or population-based) is employed when the goal is to identify the precise genetic variants related to the development of the disease (Cordell and Clayton, 2005). The objective of genetic association analysis is to identify alleles that are found more often among cases. When positive association is detected, three distinct possibilities must be considered: i) the causal allele was found; ii) the associated allele is itself associated with the causal allele, a phenomenon known as Linkage Disequilibrium (LD); in this case, the effect of the latter can indirectly be tested by genotyping the further, and iii) association is sporadic, due to chance or population stratification, i.e., the existence of cryptic differences in the genetic background of the population sample. To overcome population stratification bias, association analysis using a family-based design is recommendable. The rationale is to monitor disease allele transmission using family trios composed by the two parents and one affected child. Association is characterized if an allele is over- or under-transmitted from heterozygous parents to the affected offspring, as detected by the Transmission Disequilibrium Test (TDT).

Association analysis is the best approach to identify genes exerting a moderate to small effect over the phenotype. Usually, candidate genes are selected based on functional and/or positional cloning data. Today, association analysis can be conducted in large scale, such as in genome-wide association studies (GWA), hypothesis-generating strategies from which new, unsuspected genes can be found. These studies demand around 500,000 markers to cover the entire genome, as well as large population samples. To be certain about the markers influence, it is necessary to replicate the study in different populations.

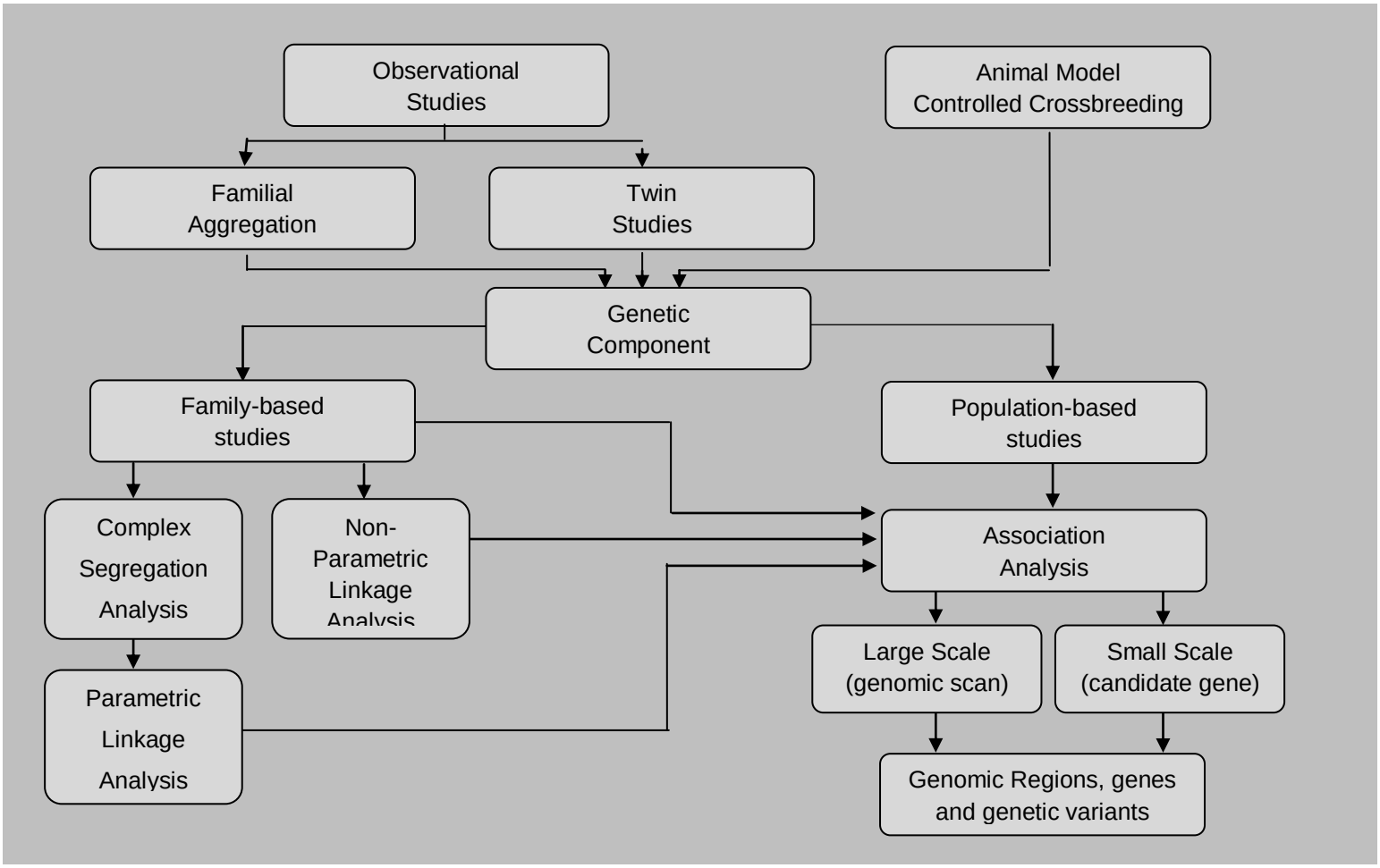

Figure 1 - Flowchart - strategies for genetic (Werneck, Mira *et al.*, 2009)

1.3 COMPLEX SEGREGATION ANALYSIS

Complex segregation analysis is a statistical method used with the objective to test if there is a major gene effect controlling disease phenotypes. A major gene is a gene exerting an effect strong enough that it can be distinguish from others, but does not assume it is the only gene involved. Also, CSA allows testing different hypothesis of transmission of the phenotype within pedigrees, such as Mendelian inheritance, taking into account the effect of non-genetic covariates and familial correlations. The hypotheses are tested using the maximum likelihood method. If a major gene is identified, it is possible to estimate parameters such as, for a quantitative phenotype, allelic frequency, genotypic means and variances.

Different models were created to analyze familial transmission using CSA, with the most common being the mixed and the regressive models. In this study, we performed CSA using the regressive model, due to advantages such as: (i) it is unnecessary to specify the genetic or environmental origin of the familial correlation; (ii) it is possible to estimate at the same time genetic and environmental parameters and test the interaction between gene and environmental covariates, and (iii) there is no difficulty of working with large pedigrees (Lathrop, Lalouel *et al.*, 1984). In addition, regressive models allow the possibility to perform analysis using quantitative phenotypes – using linear regression (Bonney, 1984) – and binary phenotypes – using the logistic model (Bonney, 1986). There are two classes of regressive models for continuous traits: Class A models assume that sibling subtypes are dependent only because of common parentage, while class D models assume that the sibling correlations are equal, but not necessarily only due to common parentage. Finally, the regressive model gives the opportunity to estimate the effects of genetic and behavior factors, as well as residual familial dependences and their interaction.

1.3.1 Regressive Methods: Principles

The regressive models will regress the individual phenotype to: (i) the effect of a major gene; (ii) to the phenotype of the preceding individuals in the family; and (iii) the covariates of interest.

1.3.1.1 Estimation of the Familial Maximum Likelihood

In the regressive Bonney model, the expression using the phenotype *Y* of the family members with *n* individuals, knowing the covariates, is:

$$f(Y|X)$$

where:

- $Y=(y_1, y_2, \ldots, y_i, \ldots, y_n)$ is the vector of the phenotypes observed, with $Y=0$ if the individual does not have the disease and 1 if the individual has the disease;

- $X=(x_1, x_2, \ldots, x_i, \ldots, x_n)$ is the vector for the covariates, where each *x* is a vector corresponding to each covariate measured in each member of the family.

For the introduction of the major gene effect, because it is not measured in the population, all possibilities are estimated and summed up. After, for each estimated possibility, its probability is appraised according to the precedent individuals in the family. The major gene is expressed as a vector:

- $G=(g_1, g_2, \ldots, g_i, \ldots, g_n)$, where g_i is the genotype of each individual.

The genotype can have 3 values (AA, AB, BB) in the case of a bialellic gene.

To formulate the maximum likelihood with a sample of families, it is necessary to convey the probability density of the observed phenotypes conditioned to the covariates:

$$f(Y|X) = \sum_{G} P(G)\, f(Y|G, X)$$

composed by two functions: the probability of jointly observing the genotypes – P(*G*) – and the probability of jointly observing the phenotype knowing the genotype and the covariates – $f(Y/G,X)$.

1.3.1.1.1 The Probability of Jointly Observing the Genotypes: P(*G*)

This probability is composed by the product of the *n* genotype probabilities of *n* individuals, each one being conditional to the genotype of the individuals who are precedent in the family, as defined by:

$$P(G) = P(g_1)\prod_{i=2}^{n} P(g_i|g_1, g_2, ..., g_{i-1}) = \prod_{i=2}^{n} p_i$$

The probability p_i of the individual genotype *i* can be defined by two ways:

- If *i* does not have parents in the studied families, so p_i is independent on the precedent genotype and is equal to p^2, 2pq or q^2 for *i* genotypes AA, AB or BB, respectively (assuming that the population is in Hardy-Weinberg equilibrium and *A* is the susceptibility allele);

- If *i* has the parents in the studied families, so p_i depends on the genotype of the parents and is equal to $P(g_i/g_p,g_m)$, which is the probability that *i* has the genotype g_i knowing the paternal (g_F) and maternal (g_M) genotypes. This probability is a function of the transmission probability τ_{AAA}, τ_{ABA}, τ_{BBA}, which is the probability of individual AA, AB or BB to transmit the allele *A* to his/her son/daughter.

In this case, the Mendelian transmission is: τ_{AAA}=1, τ_{ABA}=0.5 and τ_{BBA}=0.

1.3.1.1.2 The Probability of Jointly Observing the Phenotype Knowing the Genotype and the Covariates: $f(Y/G,X)$

It is the density function of a multinormal distribution, which is composed by the product of *n* probability densities following a normal distribution when regressing the phenotype of each subject on his/her genotype, his/her covariates vector and his/her precedent relatives phenotypes, adjusted on their genotypes and their covariates, as follows:

$$f(Y|G,X) = f(y_1|G;X)...f(y_2|g,y_1,G,X)...f(yi|y_1,...,y_{i-1},G,X)...f(y_n|y_1,...,y_{i-1},G,X)$$

The general term is: $f(y_i/y_1\ldots, y_{i-1}, G, X)$, known as z_i, and named "penetrance function", that follows a normal distribution with parameters μ_i and σ_i^2. The mean is decomposed in

$$\mu gi + \sum_{j=1}^{i-1} [\beta_{ij}(y_j - \mu_{gi} - \beta_{gi}X_j)] + \beta_{gi}X_i$$

where:

- μ_g is the mean of the individual phenotype with genotype *g* following the classic restriction $\mu_{AA} \geq \mu_{AB} \geq \mu_{BB}$ if the allele *A* predisposes to higher values of the quantitative trait;

- β_{ij} is the regression coefficient of the individual *i* phenotype (y_i) over the phenotype of his precedent *j* (y_j) adjusted by his genotype (g_i) and his covariates (x_i). This coefficient β_{ij} is expressed by using the coefficients of correlation ρ_{ij}, which can be of four types when using the class D:

    - ρ_{fm}: correlation between spouses if *i* and *j* are spouses
    - ρ_{fc}: correlation between father and child if *j* is the father of *i*
    - ρ_{mc}: correlation between mother and child if *j* is the mother of *i*

- ρ_{ss}: correlation between sibs if *i* and *j* are siblings
- ρ_{ij}=0 in the other cases

- β_g is the vector of the regression coefficients over non-genetic covariates. This coefficient can be conditioned to the genotype. Also, this coefficient allows the interaction between gene and non-genetic covariates.

In the case of a quantitative trait, the variance is also defined. The variance σ_i^2 is a function of σ_R^2 (the residual variance of the phenotype after tested the gene and the covariates effect) and the familial correlations ρ_{ij}. It is supposed that σ_R^2 is equal for each type of relatives.

1.3.1.2 Parameters Estimation and Strategies to Test the Hypothesis

Different hypothesis of transmission are estimated on a CSA, using the following parameters:

- Allele *A* frequency: $q_{A;}$
- Genotypic mean: $\mu_{AA} \geq \mu_{AB} \geq \mu_{BB}$ ($\mu_{AA}=\mu_{AB} \geq \mu_{BB}$ if *A* is dominant and $\mu_{AA} \geq \mu_{AB}=\mu_{BB}$ if *A* is recessive);
- Residual familial correlation coefficient: ρf_m, ρ_{fc} , ρ_{mc} and $\rho_{ss;}$
- Residual variance: $\sigma^2_{R;}$
- Transmission probability: τ_{AAA}, τ_{ABA} , τ_{BBA}.

Hypotheses are tested against each other using the Likelihood Ratio Test (LRT), which is the comparison between the maximum log-likelihood of the H0 and the observed H1, more general model. The two models are nested, because H0 is a particular case of H1. The -2*Ln*LRT is an asymptotic χ^2 with *n* degrees of freedom, where *n* is the difference between the number of parameters estimated in the two models. When using this method, it is possible to address the following questions, as summarized in table 1:

1. Is there an evidence for familial correlation? Here, it is necessary to compare two models: model I [model without familial correlation (H0) – sporadic] and

model II [model with estimated familial correlation (H1)]. If the model I is rejected (H0 is rejected), then it is possible to determine which kind of familial correlation exists (ρ_{fm} and/or ρ_{fc} and/or ρ_{mc} and/or ρ_{ss} and/or ρ_{ij});

2. Is there an evidence for a familial correlation effect together with a major gene effect? If in (1) familial correlation effect is maintained in the model, then it is necessary to compare model II – familial correlation (H0) – with a more general model (H1) – model IV (familial correlation plus a major gene effect). If H0 is rejected, evidence of a major gene effect is obtained and it is possible to test: (i) the dominance effect and (ii) the gene-covariate interaction;

3. Does the major gene explain all the familial correlation observed before? If model IV is kept in (2), familial correlation is removed and the new model (model III), including only the major gene effect, is tested (H0). If this test is significant, rejecting H0, model IV is considered the best-fit – the major gene alone does not explain the observed familial correlations.

4. Is the major gene effect compatible with a gene? To test this hypothesis, it is necessary to verify if the major gene effect found previously is compatible with Mendelian transmission. Consequently, two tests must be conducted:

    - Compare models IV or III (H0), which follows a Mendelian transmission (τ_{AAA}=1, τ_{ABA}=0.5 and τ_{BBA}=0), with a model of general major gene transmission (H1, model VI), which estimates the three transmission probability. A Mendelian transmission is demonstrated when H0 is not rejected;

    - Compare model V (H0, absence of transmission) with VI (H1, general transmission). The model V, absence of transmission parents-children (τ_{AAA}=τ_{ABA}=τ_{BBA}), is rejected when transmission is observed.

When models are not nested, they are compared using the Akaike criterion (Akaike, 1974). This method will penalize the models which have more

estimated parameters using: AIC=-2lnL+2 (number of estimated parameters). Therefore, the best model is always the one having minimum AIC.

Table 1 – Hypotheses in the regressive model class D

	μ_{AA}	μ_{AB}	μ_{BB}	q_A	τ_{AAB}	τ_{ABB}	τ_{BBB}	σ^2	ρ_{FM}	ρ_{FS}	ρ_{MS}	ρ_{SS}	β_X
I - Sporadic model	+	[+]	[+]	(0)	-	-	-	+	(0)	(0)	(0)	(0)	+
II - Family correlation	+	[+]	[+]	(0)	-	-	-	+	+	+	+	+	+
III - Major Gene	+	+	+	+	(1)	(0.5)	(0)	+	(0)	(0)	(0)	(0)	+
IV - Major gene and correlation	+	+	+	+	(1)	(0.5)	(0)	+	+	+	+	+	+
V - Absence of major gene	+	+	+	+	+	[+]	[+]	+	+	+	+	+	+
VI - General transmission	+	+	+	+	+	+	+	+	+	+	+	+	+

+: estimated parameter

(): fixed parameter by the hypotheses

[]: dependent parameter

-: parameter not pertinent/relevant in the hypotheses test

x: explicative covariate

1.4 GENETIC EPIDEMIOLOGY OF DENTAL DECAY

Since the 1930's, genetic research has been conducted to increase the comprehension about the genetic component associated with individual susceptibility and resistance to dental decay. These studies have been conducted following two main lines of research: molecular epidemiology of oral bacteria that colonize and contribute for the dental decay development and identification of host genetic risk factors for caries susceptibility. The main strategy when performing research with bacteria is to try to identify strains with higher carcinogenicity (Wen and Burne, 2002; Cheryl, Peter *et al.*, 2005). Host genetics is conducted using both animal models and human populations. The focus of this thesis is on human genetic susceptibility to caries development. In the following section, we present the manuscript of a review article prepared with the objective to (i) offer, to a non-geneticist readership, an overview of the main tools for genetic analysis of complex

traits; (ii) to summarize the main advances resulting from the use of these tools to investigate the genetics of dental decay. The manuscript has been recently accepted for publication at the journal “Oral Diseases” (Werneck, Mira *et al.*, 2009).

1.4.1 Review Article

A critical review: An overview of genetic influence on dental caries

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A critical review: An overview of genetic influence on dental caries

Abstract

Dental decay is a complex, chronic disease and one of the most common illnesses in dentistry today. Several dental decay risk factors have been identified during the last years; however, these variables alone may not entirely explain the disease development. Genetic research applied to dental decay began in the 1930´s with experimental reports in animals and human observational research. Only recently, have some studies begun to search for genetic polymorphisms in humans and apply linkage analysis. However, due to the complex characteristics of the disease, strong influence from several biological and environmental factors, and small number of genetic studies related to dental caries, the genetic basis still requires further study. Therefore, the aim of this review is to provide a brief description of the current methodology for genetic analysis of complex traits, followed by a comprehensive evaluation of the literature related to genetic susceptibility/resistance to dental decay and a discussion of different aspects of the applied methodology. Advances towards the elucidation of the dental decay genetic

basis may contribute to the understanding of the disease etiopathogenesis and to the identification of high risk groups, thus providing potential targets for effective screening, prevention and treatment.

Genetic Analysis of Complex Traits

Genetic human diseases are classified into two categories: (i) Mendelian diseases, and (ii) complex diseases. Mendelian diseases are rare and generally caused by variations in a single gene (monogenic). They present a perfect correlation between genotype and phenotype. A complex disease is the result of an interaction between genetic (often polygenic) and non-genetic factors (Strachan & Read, 2002, Sørensen et al., 1988). Dissecting the genetic component of a complex disease is not, however, a trivial task. Understanding the genetic basis of susceptibility to these frequent diseases could have a profound impact on public health. Examples of complex diseases with a known genetic component include infectious disorders such as leprosy (Mira et al., 2004, Moraes et al., 2006, Mira, 2006, Mira et al., 2003, Ranque et al., 2005, Alcais et al., 2007, Ranque et al., 2007), tuberculosis (Remus et al., 2004, Fieschi et al., 2003, Baghdadi et al., 2006), and oral diseases such as periodontitis (Souza, 2005, de Brito Junior et al., 2004) and dental decay (Beck & Drake, 1975, Bonecker & Cleaton-Jones, 2003, Finn & Caldwell, 1963, Conry et al., 1993, Boraas et al., 1988).

Genetic analysis of complex diseases takes into consideration all genetic and non-genetic factors that influence disease development. Generally, the first step is to determine whether or not there is a genetic component influencing the disease development (Fig. 1). At this stage, the main strategies are experimental: application of animal models and controlled crossbreeding, and observational: applicable to human populations, such as familial aggregation and twin studies. Typically, animal studies use inbred strains presenting extreme known phenotypes to analyze the result of controlled crossing, correlating genotypes and phenotypes (Kanamoto et al., 1994). Familial aggregation analysis investigates the clustering of disease cases in large pedigrees resulting from excessive sharing of genetic and/or environmental variants (Burton et al., 2005, Hopper et al., 2005). When conducting a genetic analysis, no attempt is made to determine the cause of aggregation; the sole objective is to observe clusterization. The rationale for twin studies is straightforward: monozygotic twins (MZ) share 100 % of genes and dizygotic twins (DZ) share 50 % of their genes. Importantly, twins normally share the same habits and environment

during their first years, thus, any epidemiologic influence should be minimized (Shuler, 2001, Brathall & Hänsel, 2005, Townsend et al., 2008). A sophistication of the design in twin studies is to be reared apart, generating even more powerful results (Conry et al., 1993, Boraas et al., 1988). These studies compare intra-class correlation between MZ and DZ twin pairs and/or calculate the heritability (the proportion of the phenotypic variability due to genetic variance).

To obtain genetic parameters not detected when conducting observational studies, a Complex Segregation Analysis (CSA) can be developed. CSA is a specific familial aggregation analysis, focusing on the pattern of aggregation within families (Burton et al., 2005). CSA includes an analysis of pedigrees (Hassel, 1995), and is generally carried out in advance in order to justify costly molecular studies. It is defined as a statistical methodology to identify the transmission of inherited traits for a particular phenotype using family data, and to elucidate genetic effects (Elston & Stewart, 1971). In a CSA, information regarding pedigrees and phenotype data is compared using the maximum likelihood test. The analysis includes and compares genetic mechanisms (monogenic/polygenic; dominant/recessive), allelic frequencies and penetrances, in order to obtain the best likelihood among all variables and tested models (Burton et al., 2005, Thomas, 2004). The parameters of the genetic model defined by a CSA are potentially useful in subsequent genetic mapping studies. However, the method does present some limitations, for instance it cannot produce information regarding the exact genetic nature of all genes and sequence variations involved.

DNA-based strategies for genetic epidemiology studies usually involve linkage and/or association analysis.

Linkage analyses are family-based studies designed to locate chromosome regions that may contain genes related to the study disease (Dawn Teare & Barrett, 2005). The goal is to find non-random segregation of chromosomal loci and phenotypes of the disease being mapped. If genetic and disease markers co-segregate, regions containing candidate genes for the disease are identified (Fig. 2). The two methods of conducting linkage analysis are parametric (model-based) and non-parametric (model-free). For parametric analysis, it is necessary to specify parameters of the genetic model involved, obtained from the CSA (Burton et al.,

2005). Non-parametric linkage analysis is performed when it is not possible to infer a genetic model by CSA. Statistical significance is defined by the logarithm odds (LOD) score method, reaching significance with a score of greater than 3.0 for candidate region analysis, and 3.3 for genome-wide studies (Thomas, 2004). Linkage analysis is a powerful tool to locate genomic regions exerting strong but not moderate or weak effects over the phenotype (Risch, 2000). The advantage of this approach is the fact that it allows for a genome-wide search with a relatively small number of markers (approximately 400). The main limitation of linkage analysis is that it normally locates a chromosomal region spanning across several megabases and a number of genes. In order to pinpoint the precise gene and the variants causing the genetic effect, methods with greater sensitivity, such as association analysis are required.

Association analysis (family or population-based) is applied to identify the precise genetic variants related to disease development (Cordell & Clayton, 2005). The objective of genetic association analysis is to identify the most frequent alleles When a positive association is detected, three distinct possibilities are presented: i) the causal allele was found; ii) the associated allele is itself associated to the causal allele, a phenomenon known as linkage disequilibrium (LD); in this case, the effect of the latter can be indirectly tested by genotyping the former; iii) association is sporadic due to chance or population stratification, i.e., the existence of cryptic differences in the genetic background of the population sample. To overcome population stratification bias, association analysis using a family-based design can be applied. The objective of this method is to monitor disease allele transmission using family trios composed by the two parents and one affected child. An association is identified when an allele is over or under-transmitted from heterozygous parents to the affected offspring, as detected by the transmission disequilibrium test (TDT, Fig. 3). Association analysis is the ideal approach for identifying genes that generate moderate to small effects, and can be conducted both in a small scale candidate gene study, and a large scale, genome-wide association study (GWA). In candidate gene analyses, genes are selected from functional and positional-cloning studies. In large-scale, hypothesis-generating studies, new, unsuspected genes can be found. However, these studies demand approximately 500,000 markers to cover the entire genome, as well as a large population sample. To date, the challenge of GWA is to find an adequate approach for handling and

analyzing the data and for discrimination of true association from signals generated by chance, due to the tremendous number of tests applied. Replication of findings on a series of independent populations has become an increasingly accepted strategy to validate results from GWA studies.

Dental Caries as a Complex Trait

Dental caries is a complex, chronic, multifactorial disease (Services, 2005, Fejerskov, 2004) and one of the most common diseases in Dentistry, together with periodontal disease and malocclusion (Fejerskov, 2004, Brathall & Hänsel, 2005). Dental decay has an important role in the manifestation of tooth pain and loss, and has been associated with problems in school and absenteeism in the workplace (Fejerskov, 2004, World Health Organization, 2004), leading to a decrease in quality of life (Petersen, 2003). Moreover, oral health presents a close association with the individual's general health, and may be a risk factor for several diseases (Petersen, 2003).

The index recommended by the World Health Organization (WHO) (World Health Organization, 2004) for caries estimation is the Decayed, Missing and Filled Teeth (DMFT) index (Petersen, 2003, Klein & Palmer, 1940). The DMFT index has been generally decreasing in the last few years in developed and developing countries. According to WHO (World Health Organization, 2004), the DMFT index in children of 12 years of age was higher than 3.0 in 49 % of the 184 reporting countries in 1980. In 2000, this value for children of the same age was equal to or less than 3.0 in 68 % of the same countries studied (World Health Organization, 2004), reflecting a decline of the disease over the last 20 years, even in the developing countries (Peterson, 2005). Although such information may appear satisfactory for health professionals, according to the WHO, this disease still affects from 60 to 90 % of children at school age as well as the majority of adults and is the most prevalent mouth disease in many countries (World Health Organization, 2004).

Numerous studies have shown that the global decline in prevalence of dental caries is occurring non-homogeneously throughout many countries (Bonecker & Cleaton-Jones, 2003, World Health Organization, 2004). Although strategies such as the use of topical fluoride, sealants and diet control have been developed to prevent dental decay, their effectiveness in eliminating the disease is not well established (Petersen, 2003, Dye et al., 2007). Dental caries, early tooth loss and edentulism seem to concentrate in specific groups of individuals. This phenomenon, termed *polarization* (Pine, 2005), has been exhaustively discussed, but its cause still remains obscure.

Since the 1960's, dental decay has been suggested to be the result of the interaction of four major factors: biofilm, diet, time, and host (Evans et al., 1993, Keyes, 1960, Keyes, 1962). When biofilm is exposed to highly fermentable carbohydrates, cariogenic bacteria are selected, modifying the biofilm composition (Cury et al., 2000, Nobre dos Santos et al., 2002). The main reported cariogenic bacteria are *Streptoccocus mutans (S. mutans), S. sobrinus* and some species of *Lactobacillus* (Feathrstone, 2004, Keyes, 1962). Continuous exposure to acids produced by these bacteria could lead to dental decalcification (Feathrstone, 2004). Thus, when a more cariogenic biofilm occurs and the host buffer capacity cannot compensate the acid attack of the bacteria, dental cavities may appear. Also, salivary flow and saliva composition are important to the biofilm etiopathogenicity (Lenander-Lumikari & Loimaranta, 2000). Moreover, for the past four decades, different authors have described gender, ethnicity and age as additional risk factors for the disease progress (Evans et al., 1993, Antunes & Peres, 2006).

Environmental factors, such as behavioral habits (Fejerskov, 2004), may also influence the development of dental decay. Low socioeconomical status (SES) is a non-biological risk factor which is often related to educational level, the perception of the individual about his/her own health, life style, dietary composition, and access to dental care (Antunes & Peres, 2006, Bastos et al., 2007). All these factors play a role in the development of dental caries. Hygiene habits are also correlated with the educational level and SES (Adair et al., 2004), and the frequency of tooth brushing has been shown to influence the amount of caries (Chesters et al., 1992). Access to fluoridated water another variable contributing to the decline of

tooth decay (Griffin et al., 2007, Antunes & Peres, 2006, Krasse, 1996). Family size can be considered a risk factor for dental decay: individuals from large families have a greater probability of presenting high DMFT indices (Evans et al., 1993).

However, the combination of all the factors mentioned above does not entirely explain disease outcome. Individuals exposed to the same levels of environmental risk factors present differences in the DMFT index (Pine, 2005). Those differences may be due to the fact that environmental factors can be more cariogenic for some than for others, suggesting an influence of genetic factors in the etiopathogenesis of dental caries.

Genetic Analysis of Dental Caries

Bacteria Genetic Studies

The question about the genetic influence in dental decay has been discussed since the 1920's (Bachrach & Young, 1927). From the 1970's through the 1990's the great majority of studies concerning genetic aspects of caries searched for gene variants in cariogenic bacteria (Macrina et al., 1990). The involvement of *S. mutans* and its different genotypes in susceptibility to dental decay has been widely studied, and many *S. mutans* strains have already been identified as having influence on the disease (Napimoga et al., 2005). One example involves results obtained from two studies with *S. mutans*, in which the authors identified genetic changes able to encode the proteins involved in biofilm development (Wen & Burne, 2002, Cheryl et al., 2005). These polymorphisms were associated with an increment of biofilm virulence, with impact on dental decay risk.

A more recent approach aimed to associate variables of microorganisms with a host response. For example, the relationship among human leukocyte antigen (HLA) class II and *TNFA* alleles, levels of oral bacteria that play a role in the etiology of dental caries, and the DMF surface (DMFS) index was investigated in Afro-American women (Acton et al., 1999). The results support the

hypothesis of an association between host HLA class II and *TNFA* genetic profile with colonization of *S. mutans*, *L. casei*, and *L. acidophilus*.

Host Genetic Studies

Genetic studies have been conducted to better comprehend the genetic component associated with the individual susceptibility to dental decay development. The approaches which have been used include: i) experimental studies involving animal models and controlled crossbreeding (Kanamoto et al., 1994, Hunt et al., 1944, Hunt et al., 1955); ii) observational studies involving human populations, such as familial aggregation analysis (Garn et al., 1976a, Garn et al., 1976b) and twin studies (Conry et al., 1993, Finn & Caldwell, 1963, Hassel, 1995, Boraas et al., 1988, Shuler, 2001, Sofaer, 1993), and iii) linkage and association studies (Bagherian et al., 2008, Deeley et al., 2008, Patir et al., 2008, Slayton et al., 2005, Vieira et al., 2008).

Experimental Studies in Animals

Studies with different mice strains support the hypothesis that differences in susceptibility to caries could be due to hereditary factors (Steggerda & Hill, 1936). The observation that some mice from genetically heterogeneous populations differed in the disease experience under the same environmental conditions suggested the existence of dental decay susceptibility (Hunt et al., 1944) and resistance (Hunt et al., 1955). Animals strains are selected and crossed based on their level of susceptibility/resistance to disease so that the trait can be traced over the next generations. In the classic Hunt-Hoppert studies (Hunt et al., 1955, Hunt et al., 1944), 35 days were necessary for the development of carious lesions in the susceptible strain as opposed to 505 days for the same effect in the resistant strain. These results strongly suggest the influence of genetic differences between mice strains in controlling caries progression. In a Harvard study also comparing

susceptible and resistant lines, caries lesions were almost ten times more extensive in susceptible mice than in resistant animals at the age of 110 days (Willett et al., 1958). However, when the mice were exposed to a high cariogenic diet, the difference between the two lines decreased (Larson, 1965, Larson et al., 1968). In the 90's, Kanamoto *et al.* (Kanamoto et al., 1994) also demonstrated the genetic influence in caries scores of molar teeth, when comparing four inbred strains of male mice which were inoculated with *S. mutans* and fed with a cariogenic diet. Other studies started to identify genomic regions and polymorphisms related to susceptibility and/or resistance variations (Kurihara et al., 1991, Quivey et al., 2005). Research on mice showed linkage between dental decay and chromosome 2, 8 (Nariyama et al., 2004) and 17 (Suzuki, 1998), where the mice MHC complex is localized. Matsumoto *et al.* suggested that the E2f1 gene, whose mutation cause a decreased volume of saliva production and protein production rate, affected susceptibility for oral biofilm formation by streptococci (Matsumoto et al., 2004). A study with Aqp-/- knockout mice showed a relationship between this gene and the reduction of salivary flow, as well as an increase in caries, mainly in buccal and succal surfaces (Culp et al., 2005). Finally, another study comparing MRL/l and MRL/n strain mice, for which the former possess a lymphoproliferative gene inducing swelling of systemic lymph nodes, investigated whether the salivary immune response caused by *S. mutans* infection prevented dental caries in the two strains. The results showed a difference between strains, indicating that the salivary immune response may be an important factor in regulating dental decay development (Maeda et al., 1995).

Observational Studies

Familial Aggregation Studies

Several studies of familial aggregation in caries have been reported since the 1930's, as observed in table 1. As a result, a solid body of evidence was created indicating familial clustering of caries experience and allowing for speculation whether or not there are genetic factors controlling the disease. One of the first studies, reported in 1938, investigated dental decay correlation in siblings (Klein &

Palmer, 1938). Students at 10 years old were classified into two groups: no caries and having a DMFT of six or more. The siblings of caries-free children had lower average caries scores than the siblings of the susceptible children. A larger study involving 16,000 sibpair participants in the Ten-State Survey was conducted. DMFT correlation was estimated and gender, ethnicity and age (ranging from 7 to 18 years old) were used for stratification. Sibling correlation was found (r = 0.23 to 0.41), presenting higher correlations between black and older sibpairs, however no evidence was described for ethnicity influence (Garn et al., 1976a).

Investigations were also conducted to observe familial aggregation and the relationship to caries between parents and children. A study in 1953 collected data from caries-free and no caries-free males enlisted for military service and related individuals. Parents and siblings of the caries-free subjects had a significantly lower caries index than the parents and siblings of the non caries-free group (Book & Grahnen, 1953). In a natural fluoride area, children reflected the parents' caries experience in a study including 5,400 individuals. Even with the fluoride exposure, both groups (high and low level of caries) had differences in the degree of susceptibility, demonstrating that fluoride does not decrease the genetic risk (Klein, 1946). A recent study conducted in Quebec with mother-child pairs composed of 6,039 dentate and 264 edentulous mothers showed that children from the latter were more likely to experience caries on both primary and permanent dentitions when compared with children of dentate mothers. In the same study, environmental factors (socio-economic status, age, gender, and children's oral-health-related behaviors) were also assessed but did not show significant influence (Bedos et al., 2005).

In contrast, the observation that spouse-pairs share similar DMFT scores suggests that variables such as household, diet and environmental stress are also determinants for the outcome of caries disease (Garn et al., 1977).

Twin Studies

Numerous twin studies for caries have been reported, as observed in table 1. High concordance rates between MZ twins for several dental phenotypes

such as dental decay, tooth size, dental arch dimensions, intercuspal distances and occlusal traits have been described (Townsend et al., 2008, Townsend et al., 2003). Howoritz *et al.* (Horowitz et al., 1958), using matched pairs of MZ and DZ twins, concluded that MZ twins had greater caries concordance ($p < 0.001$). In 1979, Fairpo (Fairpo, 1979), working with 100 MZ and 120 DZ twin pairs, concluded that there was genetic influence in the susceptibility to dental decay development in both deciduous and permanent teeth. In 2005, two studies using twin pairs estimated heritability, which measures the percentage of the phenotypic variance that is the result of genetic factors. Bretz *et al*. (Bretz et al., 2005a) studied 388 twin pairs and heritability was estimated in 70 % for the dental decay. The same population was studied once more after 12 months and the heritability value was again significant (H = 30 % for the younger and 46.3 % for the older twin pairs) (Bretz et al., 2005b). The higher concordance and heritability between MZ than DZ twins may demonstrate that there is a genetic factor influencing dental decay development; however, these results do not discard the influence of environmental factors. An alternative approach in twin studies in order to dissect the environmental component of heritability is to study twins who have been reared apart. This allows a more precise assessment of the inherited component controlling the phenotype. In caries, two twin studies using twin pairs reared apart demonstrated that MZ twins had higher similarity in incidence of dental decay than DZ twins, despite the fact that the individuals have been raised in different families, communities and/or even countries, a strong argument in favor of the existence of a inherited contribution (Boraas et al., 1988, Conry et al., 1993). Another advantage of these studies was that the patients' average age was over forty years old and all pairs had been separated shortly after birth.

In contrast, there are twin studies for which the results do not show significantly higher concordance rates for caries occurrence between MZ versus DZ pairs. Comparison between 82 pairs of female-female twins from 6 to 12 years showed strong evidence of genetic influence controlling third molar presence, tooth size, arch size, and upper lateral incisor malformation; while a weak heritability was seen in tooth eruption and caries (Liu et al., 1998). Bordoni *et al*. worked with a sample of 17 MZ twins and 17 unrelated controls and concluded that a genetic component is more important in tooth morphology and eruption timing than caries (Bordoni et al., 1973). In addition, a study with 280 pairs of twins demonstrated a

higher concordance rate for caries in MZ twins, but the results were not statistically significant (Gao, 1990).

Linkage and Association Analysis

Animal Studies

More recently, the aim of host genetic studies using animal models has shifted towards the identification of the polymorphisms associated with susceptibility and/or resistance (Kurihara et al., 1991, Quivey et al., 2005), and several candidate genomic regions and genes have been identified. Genetic linkage between dental decay and *loci* of chromosomes 2, 8 (Nariyama et al., 2004) and 17 (Suzuki, 1998), where mice major histocompatibility complex (MHC) is localized, was identified. A comparison between wild type and knockout mice for the *Mrl, a* lymphoproliferative gene that induces systemic swelling of lymph nodes, showed higher levels of caries disease in the knockout animals, indicating that the immune response may be an important factor in regulating dental decay development (Maeda et al., 1995). Caries-free monkeys showed increased serum antibody titres and proliferation of T lymphocytes when stimulated with *Streptococcus* (Lamb et al., 1980). Moreover, in caries resistant subjects, a lower dose of *Streptococcus* is necessary to stimulate T-helper activity (Lehner et al., 1981). The low-dose feature was associated with the allele HLA-DRw while the high-dose was associated with HLA-DR4.

Human Studies

A polygenic nature for the genetic control of caries disease has been discussed since the 1970's. Mühlemann, in 1972 (Muhlemann, 1972), suggested that a set of genes could influence the enamel resistance while a different set could influence the saliva composition and host response to infection. Nevertheless, only recently linkage and association studies have begun to be conducted in an attempt to identify genomic regions and polymorphisms related to dental caries.

The first linkage analysis for dental decay was carried out in 2008. A genome-wide scan (392 markers) was performed aiming to identify genomic regions that might contain genes related to dental decay. The population was composed of 46 families (624 individuals), living in the same area in the Philippines. Five suggestive *loci* were identified: 3 for low caries susceptibility (5q13.3, 14q11.2, and Xq27.1) and 2 for high caries susceptibility (13q31.1 and 14q24.3) (Vieira et al., 2008). The authors highlight the presence of genes related to saliva flow control and diet preferences in regions 13q31.1, 14q24.3 and 14q11.2. Unfortunately, the authors applied parametric linkage analysis using parameters from a CSA for which details were not included in the report.

Although studies associating dental decay with genetic polymorphisms were initiated during the 1980's using animal models such as monkeys and mice, only in 2005 were the first studies involving humans published.

Genes related to enamel development and mineralization: *amelogenin (AMELX*), *ameloblastin* (*AMBN*), *tuftelin* (*TUFT1*), *enamelin* (*ENAM*), *tuftelin-interacting protein* (*TFIP11*), and *kallikrein 4* (*KLK4*) have been investigated (Slayton et al., 2005). Markers of these candidate genes were tested for association following a case-control design using a sample of children from 3 to 5 years old. No evidence for association between caries occurrence and any independent investigated gene was observed. However, when performing a multivariate analysis, the effect of the *TUFT1* gene combined with the effect of high level of *S. mutans* increased the susceptibility to dental decay (Slayton et al., 2005). Another study also investigated *AMELX, AMBN, TUFT1, ENAM, and TFIP11* for association with caries in a population from Guatemala. Strong evidence (P = 0.0000001) for association was found for one *AMELX* marker with higher DMFT (DMFT ≥ 20) and increased age-adjusted caries experience (Deeley et al., 2008). The same gene was studied in a population sample from Istanbul and the findings confirmed the previous study (Patir et al., 2008). The authors concluded that the best-fitting model for increased dmfs is composed of a combined overrepresentation of specific alleles of a marker of *TUFT1* and a marker of *AMELX* in the case group.

A study using a population-based design investigated the influence of *mannose-binding lectin* (*MBL*) gene, which plays a critical role in the immune

response in early childhood. Decreased blood levels of MBL may cause predisposition to infections and autoimmune diseases (Pehlivan et al., 2005). Polymorphisms in the *MBL* gene were analyzed and the overall genotype distribution did not significantly differ between caries-free and children with carious teeth. A different study investigated the association of HLA-DRB1 and HLA-DQB1 alleles with susceptibility to early childhood caries (ECC), a type of caries that affects the deciduous teeth of infants and toddlers. The authors found a significant association between HLA-DRB1*04 and ECC (Bagherian et al., 2008). Yu *et al.* (Yu et al., 1986) found an association between the DMFS increase and saliva levels of a specific *proline-rich protein* gene (*PRPs*), a saliva component that influences the attachment of bacteria. A subsequent study investigating a gene related with the PRPs observed an association between dental decay and the Db allele, one of the three alleles of *PRH1* gene (Zakhary et al., 2007). The same study showed that Db negative Caucasians had significantly more caries than Afro-American Db negative patients, demonstrating the importance of ethnicity associated with genetic information. Another study involved carbonic anhydrase VI (*CA6*), a secreted enzyme that catalyzes the hydration of carbon hydroxide in saliva and other body fluids. The authors found no association between the alleles and genotype distribution of three polymorphisms in the coding sequences of *CA6* gene with caries experience. However, a positive association between buffer capacity and the rs2274327 (C/T) polymorphism was found (Peres et al., 2009). Finally, a study involving a population sample of children, tested for an association between abscess or fistula formation (AFF), which may be caused when caries progresses into pulpal inflammation, with a polymorphism in the bacterial ligand CD14 (-260), an immune factor responsible for modulating the immune response. A CD14 genotype was significantly associated with the presence of 4 or more carious lesions (De Soet et al., 2008).

Genetic Analysis of Dental Decay - Future Perspectives

Several biological and environmental risk factors for dental decay development have been identified in the last few years. It is well known that dental decay is primarily determined by environmental factors; however, despite the use of different strategies to control these factors and prevent disease, caries is far from being controlled as a public health problem: dental decay continues to affect children and adults in both developed and developing countries, being one of the most important and prevalent mouth diseases (Brathall & Hänsel, 2005, Burt, 1998, World Health Organization, 2004). The characterization of high-risk individuals (Pine, 2005) may indicate that dental decay outcome can also be influenced by additional variables, such as the genetic background of the host.

When studying genetics of complex diseases, usually the first goal is to characterize the existence of a genetic component controlling phenotypes of the disease so that further comprehensive studies can be developed (Abel & Demenais, 1988, Abel et al., 1995, Feitosa et al., 1995, Haile et al., 1985, Wagener et al., 1988). Classic experimental studies using animal models have been effectively employed to demonstrate a genetic influence on caries outcomes, with differences between susceptible and resistant strains (Hunt et al., 1944, Hunt et al., 1955). Observational studies in humans have identified a genetic impact over dental decay. Reports on individuals from the same family show that the correlation of the dental decay index between parents and siblings, as well as between sibpairs, follows a pattern; therefore, the disease does aggregate in families (Garn et al., 1976a, Garn et al., 1976b, Garn et al., 1977). Twin studies have demonstrated that the concordance rate for caries occurrence increases as grows up the proportion of genome sharing between two individuals (Bordoni et al., 1973, Bretz et al., 2005a, Bretz et al., 2005b), even when the twins are reared apart (Boraas et al., 1988, Conry et al., 1993). All of these experimental and observational studies strengthen the hypothesis of a genetic influence in dental decay development. However, in the era of molecular genetics and genome-wide association studies, few advances have been made towards the dissection of the exact nature of the genetic component controlling susceptibility to dental caries. For example, to date, no confident heritage pattern has been estimated for dental caries. In this context, a well conducted CSA seems imperative to provide the first formal set of data to define the genetic inheritance model for caries, and to determine the best approaches for further genetic studies.

Moreover, this genetic model could be included in parametric linkage analysis, a powerful hypothesis-generating tool that could indicate the genomic location of major loci controlling susceptibility to disease. Finally, a CSA would include not only genetic factors but also covariables such as SES, dental hygiene, dietary composition, which for a disease as complex as caries, may be of extreme importance.

To date, one single study has used both parametric and non-parametric linkage analysis to detect genomic regions containing candidate genes for dental caries. Unfortunately, the authors did not include the results of the CSA used for parametric linkage analysis in the report (Vieira et al., 2008). Nonetheless, for a protocol widely accepted today, additional studies have yet to be conducted to replicate these first findings in different populations.

More recently, case-control association studies have been conducted to investigate candidate genes that may influence dental caries susceptibility and resistance. These studies have mainly focused on genes influencing enamel formation, saliva composition, and immune response. Nevertheless, the great majority of these findings were still not replicated. In this context, family-based association could also be used, aiming to avoid bias caused by population stratification that may remain unnoticed in a case-control design. As the most powerful tool to identify genes associated with disease available nowadays, GWA could also be applied to dental decay phenotypes, using information of very large sets of markers covering the entire genome, selected from the 6 million SNPs available at public databases (NCBI, 2009).

Identifying the genes that play a role in controlling caries susceptibility is essential for a full understanding of the molecular basis of the disease pathogenesis, and would have potential impact on the development of new preventive and therapeutic strategies – such as molecular vaccines and even gene therapy. Clinicians would be able to screen and identify susceptible patients, adopting individual, tailor-made intervention with a potential high impact over maintenance and preservation of individual oral health. Finally, the identification of genetic risk factors for caries would help reduce costs associated with treatment and prevention of one of the most frequent oral diseases.

Another discussion that should not be overlooked regarding etiopathological risk factors for dental caries is the definition of the ideal phenotype. Are the current methods of caries identification suitable for genetic studies? Further studies should be conducted in order to compare the existing and new methods of caries diagnosis to make the phenotype more precise. Moreover, the selection of individuals with high susceptibility (for example, having high DMFT and low sucrose consumption) and with low susceptibility to caries (for instance, having low DMFT and high sucrose consumption) could contribute to a more precise phenotype, and likely have stronger genetic influence than the general population.

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Fig 1. Flowchart - strategies for genetic epidemiologic investigation.

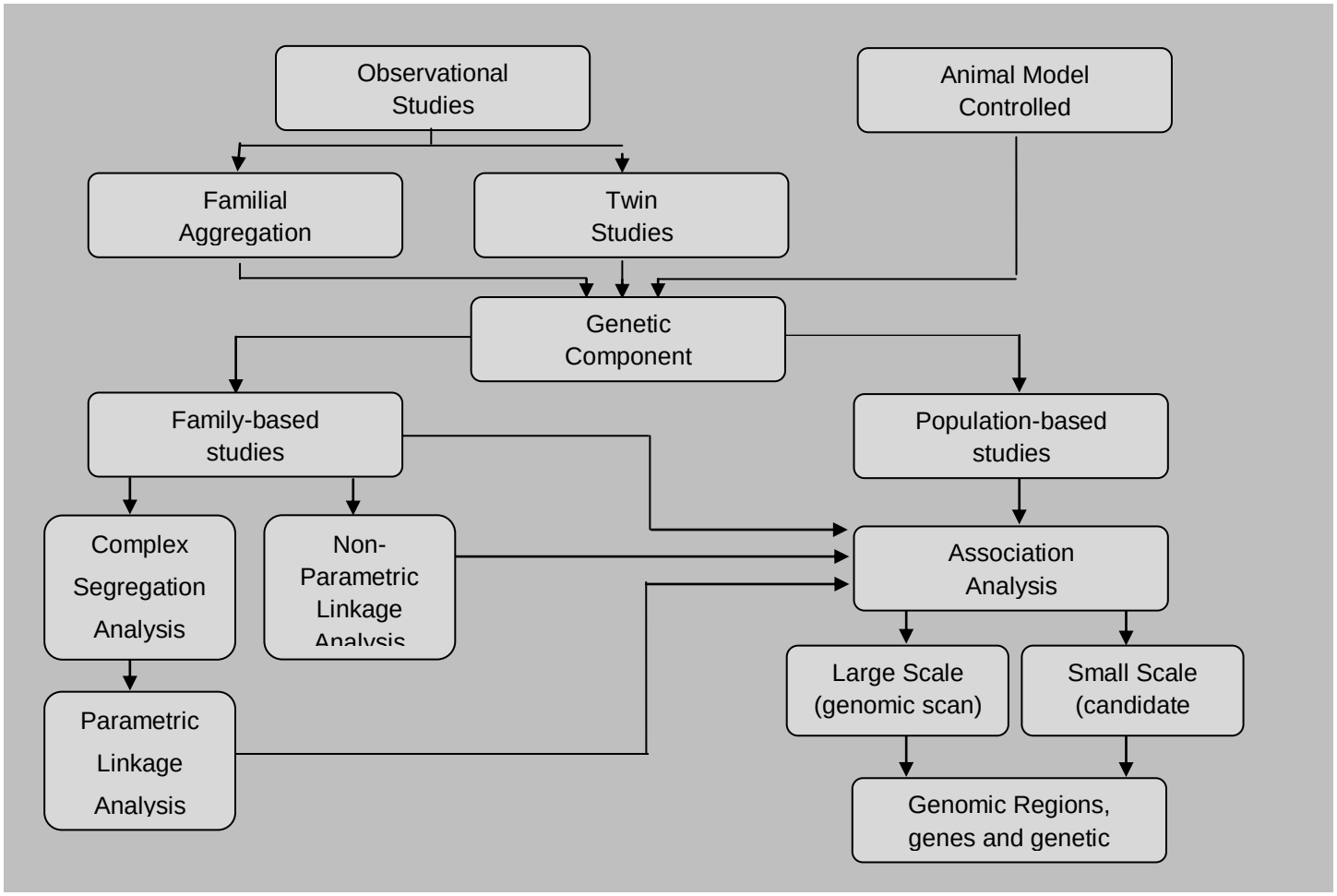

Fig 2. Linkage analysis - non-random segregation between the disease locus (T2) and marker whose location is known (g).

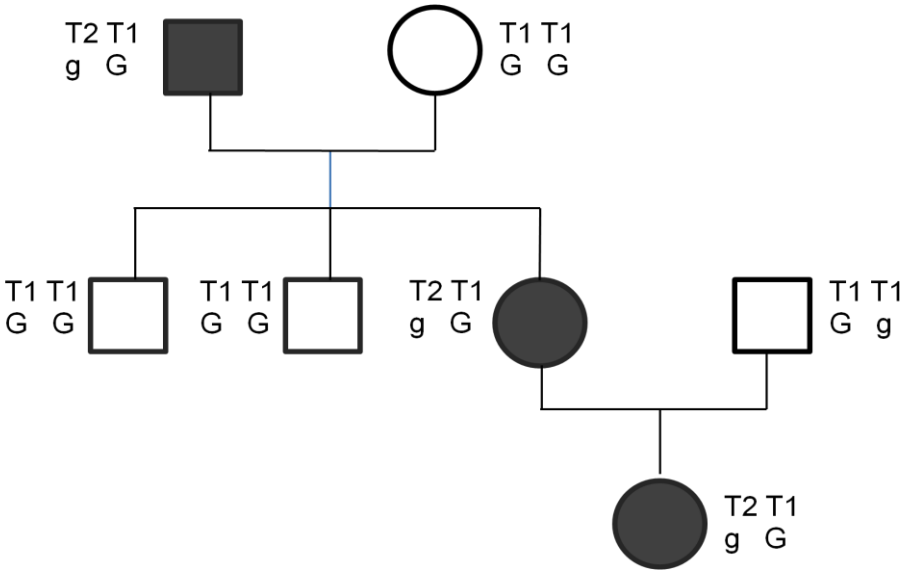

Fig 3. Transmission disequilibrium test (TDT) observes the number of times the heterozygous father Dd transmits the allele D or d to her daughter.

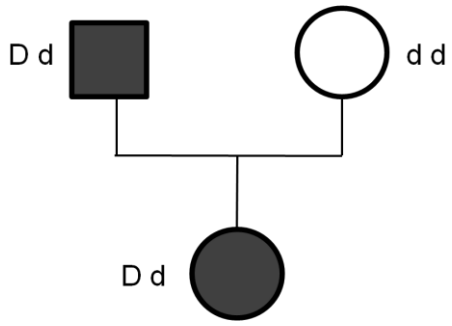

Table I. Evidence for genetic influence to dental decay susceptibility through observational studies.

Reference	Study Population (N)	Findings
(Klein & Palmer, 1938)	Siblings (4416)	Similarity in caries rate between siblings.
(Klein, 1946)	Parents and Children (5400)	Offspring dental disease quantitatively related to parents experience.
(Klein, 1947)	Parents and Children (-)	Similarity in caries rate between parents and children.
(Book & Grahnen, 1953)	Parents and Siblings (317)	Correlation between siblings and parents of caries-free individuals.
(Garn et al., 1976b)	Parents and Children (6580)	Mother-child similarities in the DMFT scores are systematically higher than father-child.
(Garn et al., 1976a)	Siblings (16000)	Positive siblings correlation.
(Garn et al., 1977)	Spouse Pairs (1800)	Positive spouses DMFT correlation.
(Maciel et al., 2001)	Mothers and Children (-)	Positive mother and children correlation in relation to patterns of sweetness preference and caries experience.
(Bedos et al., 2005)	Mother and Child (-)	Positive correlation between edentulous mother and their children.
(Bachrach & Young, 1927)	MZ (130) DZ (171)	No differences between the MZ and DZ twin pairs.
(Horowitz et al., 1958)	MZ (30) DZ (19)	MZ more alike caries experience than DZ twin pairs.
(Mansbridge, 1959)	MZ (96) DZ (128)	MZ twin pairs with greater similarity in caries experience.
(Goodman et al., 1959)	MZ (19) DZ (19)	Intrapair variance of DZ greater than MZ.
(Finn & Caldwell, 1963)	MZ (35) DZ (31)	MZ and DZ differences greater for smooth surface caries in anterior teeth.
(Bordoni et al., 1973)	MZ (17) Unrelated Controls (-)	Greater similarity in tooth morphology and eruption timing in primary teeth between MZ than unrelated controls.
(Gao, 1990)	MZ and DZ (280)	Higher correlation in MZ, but not statistically significant.
(Conry et al.,	MZ (46) DZ (22) reared	MZ with greater within-pair similarity than DZ pairs for:

1993)	apart	teeth present, teeth present excluding third molars, teeth restored, teeth restored index, surfaces restored, surfaces restored index and surfaces restored or carious, in reared apart twin pairs.
(Boraas et al., 1988)	MZ and DZ (44) reared apart	Resemblance within MZ for number of teeth present, percentage of teeth and surfaces restored, percentage of teeth and surfaces restored or carious, tooth size, and malalignment.
(Liu et al., 1998)	MZ and DZ (82)	Strong evidence of genetic influence to third molar presence, tooth size, arch size, and upper lateral incisor malformation.
(Bretz et al., 2005a)	MZ (142) DZ (246)	For surface-based caries prevalence rates the heritability was strong - 76.3; for lesion severity the heritability was also strong - 70.6.
(Bretz et al., 2005b)	MZ (112) DZ (202)	For surface-based caries prevalence rates the heritability was moderate (H = 30.0) and greatest for the oldest groups (H = 46.3); for lesion severity the heritability was also moderate (H = 36.1) and greatest for the youngest group (H = 51.2).

Table II. Evidence for genetic influence to dental decay susceptibility through linkage and association study.

Reference	Study Population (N)/Type of Study	Candidate region(s)/gene(s)	Findings
(Slayton et al., 2005)	Children dmfs > 4 (92) and dmfs = 0 (343) / Case-Control Population-Based	*AMELX*, *AMBN*, *TUFT1, ENAM*, *TFIP11*, and *KLK4*	Tuftelin gene and high level of *S. mutans*, associated with susceptibility to dental decay.
(Pehlivan et al., 2005)	Children caries-free (40) and with carious teeth (42) / Case-Control Population-Based	MBL	No significant difference between two groups and genotypes distribution.
(Zakhary et al., 2007)	Adult Caucasians (60); Children of: Caucasian Parentage (89), African-American Parentage (96), and Mixed Parentage (23) / Case-Control Population-Based	PRH1 locus (Db)	Db-negative Caucasians had significantly more caries.
(Bagherian et al., 2008)	ECC children (44) and Caries-free children (35) / Case-Control Population-Based	HLA-DRB1 and HLA-DQB1	HLA-DRB1*04 was associated with ECC susceptibility.
(Deeley et al., 2008)	DMFT ≤ 2 (44) and DMFT ≥ 3 (66) / Case-Control Population-Based	*AMELX*, *AMBN*, *TUFT1*, *ENAM*, and *TFIP11*	Strong association of AMELX with DMFT ≥ 20 and increased age-adjusted.
(Patir et al., 2008)	dmfs ≥ 4 (91) and dmfs = 0 (82) / Case-Control Population-Based	*AMELX*, *AMBN*, *TUFT1*, *ENAM*, and *TFIP11*	TUFT1 overrepresentation of T allele and AMELX overrepresentation of the C allele.
(De Soet et al., 2008)	5 groups: caries free (53); full dental treatment (75); extraction only (66); ART filling only (77); and no treatment (77) / Case-Control Population-Based	*CD14*-260	Protection effect of the *CD14*-260 TT genotype for AFF in children with dmft+DMFT ≥ 4 at baseline.
(Peres et al., 2009)	Children (245) caries free and with caries Case-Control Population-Based.	*CA6*	Positive association between buffer capacity and the rs2274327 (C/T) polymorphism.
(Vieira et al., 2008)	46 families / Genome-Wide Linkage Analysis	--	Five suggestive *loci* were identified: - 3 for low caries susceptibility (5q13.3, 14q11.2, and Xq27.1) - 2 for high caries susceptibility (13q31.1 and 14q24.3).

2. RATIONALE AND HYPOTHESIS

Dental caries is a complex, chronic, multifactorial disease and still one of the most common diseases in Dentistry, being an important Public Health concern. Non-genetic predictive factors (determinants and modifiers) have been extensively discussed in the literature and preventive treatments have been designed based on these findings. However, recently studies have identified the existence of a major genetic component controlling dental decay susceptibility in human populations. Although exciting, these genetic studies fail to provide a description of the genetic model of inheritance (dominant/co-dominant/recessive) involved, allelic frequencies, genotype means and variance, as obtained through CSA. The parameters of the genetic model defined by CSA will be of great use in subsequent, genetic mapping studies. Our hypothesis is that there is a major gene effect controlling host susceptibility to dental decay, strong enough to be detected in a CSA.

3. OBJECTIVES

3.1 GENERAL OBJECTIVE

To describe the model of inheritance that better fits into the segregation pattern observed for caries in a collection of multigenerational families from an isolated population from north of Brazil.

3.2 SPECIFIC OBJECTIVES

I. To describe dental decay-related clinical and epidemiological variables of the study population;

II. To test the clinical and epidemiological variables for association with caries in the sample population;

III. To investigate the existence of a major gene effect controlling DMFT and decayed teeth (DT) phenotypes.

4. ORIGINAL ARTICLE

A Major Gene Effect Controls Resistance to Dental Decay in Brazil

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ABSTRACT

Despite recent advances revealing genetic factors influencing caries susceptibility, questions regarding the model of inheritance involved are yet to be addressed. We conducted a Complex Segregation Analysis on number of Decayed Teeth (DT) in a sample of homogenous, isolated families recruited at the Brazilian Amazon. A dominant, major gene effect controlling resistance to phenotype was detected. The frequency of the resistance allele “A” was 0.63; mean DT was 1.53 and 9.53 for genotypes AA/AB and BB respectively. These results are an important step towards a description of the exact nature of the genetic risk factors controlling human susceptibility to caries.

INTRODUCTION

Dental decay (or caries), one of the most common diseases in Dentistry (Fejerskov, 2004), is a chronic, multifactorial disease that exerts enormous impact on public health systems of industrialized and developing countries (Petersen, 2003). Dental decay is an important cause of dental loss and dental pain, both conditions associated with impaired performance in school and absenteeism at work (Fejerskov, 2004; Petersen, 2003), ultimately leading to a decrease in quality of life (Petersen, 2003). Even though reports have shown that the Decayed, Missing and Filled Teeth (DMFT) index, commonly used as an estimate of dental decay, has been decreasing over the last few years in developed and developing countries, dental decay continues to affect 60-90% of children at school age and the majority of adults (Petersen, 2003).

It is widely accepted that the occurrence of dental decay depends on environmental and host-related factors (Antunes and Peres, 2006; Evans *et al.*, 1993; Feathrstone, 2004; Keyes, 1960; Pine, 2005). When the biofilm is exposed to highly fermentable carbohydrates, cariogenic bacteria like *Streptoccocus mutans (S. mutans), S. sobrinus* and some species of *Lactobacillus* (Keyes, 1960) are selected. Continuous exposure to acids produced by these bacteria, associated with a limited buffering capacity of the host, leads to dental decalcification (Feathrstone, 2004). The process is modified by environmental factors, such as oral hygiene, fluoride exposure, as well as socioeconomical status (SES), gender, ethnicity and age (Antunes and Peres, 2006; Evans *et al.*, 1993). Moreover, dental caries, early dental loss and edentulism seem to concentrate in some groups of individuals (Petersen, 2003), phenomenon known as *polarization* (Pine, 2005).

Although previous studies (Ozturk *et al.*, 2010; Werneck *et al.*, 2010) point to the existence of a genetic component controlling host susceptibility to dental decay, no description of the genetic model involved, as provided by Complex Segregation Analyses (CSA), has been produced to date. The approach enables identification of a major gene effect (that is, an effect important enough to be distinguished from other susceptibility genes) and the estimation of the allele frequency and penetrance of the deleterious allele, among other parameters. Here we present the results of a CSA for dental decay performed in a sample of the Colony of Santo Antônio do Prata (the Prata Colony), a highly exposed, isolated community sharing very homogenous non-genetic variables, located in the Amazonian state of Pará, north of Brazil.

MATERIAL AND METHODS

Study population and enrollment strategy

The study was approved by the Research Ethics Committee of the Pontifical Catholic University of Paraná. All families included in the study have been recruited from the Colony of Santo Antônio do Prata, a former leprosy colony created in the early 20's with the objective to isolate leprosy affected individuals. Isolation was compulsory up to 1962; however, the population of the colony remains isolated up to date, probably due to the strong stigma associated with leprosy, a disease still highly prevalent within the community. Previous assessment indicated very homogenous environmental and socioeconomic variables and predominance of a mixed ethnic group, as reported elsewhere (Lázaro *et al.*, 2010). Family recruitment was performed using a systematic

approach aiming to reduce ascertainment bias. First, one household was randomly selected and all members were invited to participate. Upon agreement, all individuals from this first nuclear family were asked to report if there was any relative living in the colony; if yes, these relatives and their families were also contacted and included in the study. The procedure was repeated until no additional relatives belonging to this first extended family were reported. Then, the next nuclear family was selected counting two households to the left from the first included nuclear family and submitted to the same enrollment strategy. The same was applied until complete recruitment of the population sample.

Phenotype definition and epidemiological data collection

Data collection was composed of a structured interview and a clinical examination, both performed by a single investigator (R.I.W.). Prior to data collection, all individuals were asked to sign an informed consent agreeing to participate in the study. Consent regarding individuals under 18 years old was provided by the parents. The examination was conducted in the field, using natural light, tongue depressor and gauze. A case of caries was defined according to World Health Organization guidelines (WHO, 1997). Information regarding demographic characteristics (gender, age, ethnicity), SES (socioeconomic status, educational level, water supply), oral health (dietary habits, brushing habits, frequency of dental appointments) and clinical evaluation (decayed teeth, gingivitis, plaque and leprosy status) was obtained and analyzed prior to the CSA.

Statistical methods

The phenotype “number of decayed teeth (DT)” was investigated as a continuous trait. To bring the observed distributions closer to normality, DT was root transformed. Prior to the CSA, impact of non-genetic covariates on DT was investigated by univariate and multivariate linear regression analysis, as implemented in the *t*-test, correlation and linear regression analysis of the SPSS software (version 13.0). Covariates yielding statistically significant associations with DT in the multivariate analysis were included in the corresponding CSA.

Complex Segregation Analysis was conducted following the same regressive model applied recently for this population and leprosy (Bonney, 1986; Lázaro *et al.*, 2010). In the first model, sporadic transmission (Table 2, model I) includes only the non-genetic covariates with significant impact over disease susceptibility. Next, in addition to the significant covariates, the dependence on phenotypes of preceding relatives, which is parameterized in terms of familial correlations (Table 2, model II) are included in the model using the class D pattern of familial correlations (FC) (Lázaro *et al.*, 2010). Four types of phenotypic FC were considered: father-mother (FM), father-offspring (FO), mother-offspring (MO), and sib-sib (SS), with corresponding regression coefficients denoted as “ρ_{FM},” “ρ_{FO},” “ρ_{MO},” and “ρ_{SS},” respectively. To rule out the possibility of significant familial dependency due to unmeasured shared environmental factors, the next step is to include a major gene (MG) effect in the model (Table 2, model III and IV). The estimated parameters of the MG were *q* (the frequency of allele *A* predisposing to DT), μ_{AA}, μ_{AB}, and μ_{BB} (the phenotypic means of individuals with genotypes AA, AB and BB, respectively), and σ^2 (the common residual variance of the phenotype). Finally, two additional models including a MG effect were considered: (i) an “absence of

transmission" model (model V) in which three types of individuals - AA, AB and BB - are specified but in which absence of parents-offspring transmission is obtained by setting τ_{AAA},= τ_{ABA}= τ_{BBA}; and (ii) a more general transmission model (model VI) in which the three τs are estimated (Elston and Stewart, 1971). Segregation of a MG can be inferred if we fail to reject the Mendelian transmission of the major effect when compared with the general transmission model and we reject the non-transmission hypothesis when compared with the general transmission model (this latter test rules out the possibility that the failure to reject the Mendelian transmission of the major effect was due to lack of power) (Demenais *et al.*, 1986).

Parameter estimation and hypothesis testing were performed using classic likelihood strategies. Because of the study design – exhaustive reconstruction and data collection of randomly selected (instead of proband-based selected) extended pedigrees – there was no need for ascertainment correction (Tai and Hsiao, 2001). Complex Segregation Analysis was performed as implemented in S.A.G.E. (Statistical Analysis for Genetic Epidemiology) release 5.4.1 (Case Western Reserve University), using the segregation analysis program SEGREG (Sorant *et al.*, 1994).

RESULTS

Family sample and phenotype distribution

A total of 451 individuals were enrolled in the study, distributed in 11 extended pedigrees. The male/female ratio was 1.01, the mean age at enrollment was 30.72 (±16.31) years old and mean DT was 2.44.

Analysis of covariates

Univariate analysis revealed effect of age, snack habit, gingivitis and number of teeth over DT (Table 1). Multivariate linear regression analysis confirmed impact of age (p=0.02) and gingivitis (p=0.0002): dental decay was significantly more frequent among individuals with gingivitis (mean DT=1.44) when compared to individuals without gingivitis (mean DT=0.98; p=0.0004). As demonstrated in figure 1, the mean DT reached the highest value at the age class of 20-29 years old.

Complex Segregation Analysis

Result of CSA is shown on table 2. Since correlations between spouses (ρ_{FM}), father-offspring (ρ_{FO}), and mother-offspring (ρ_{MO}) were not observed on CSA, these parameters were not included in the table. There was evidence of FC since the sporadic model without FC was rejected against the model that included SS correlation [model I vs II, χ_2(1df)=4.56 p=0.03]. The inclusion of a dominant major effect to SS correlations notably improved the fitness of the model [model II vs III, χ_2(3df)=100 p=10^{-21}]. Interestingly, removal of residual SS correlations did not significantly impact on the fitness [model III vs IV, χ_2(2df)=0.62 p=0.73]. Finally, the transmission of the dominant major effect was compatible with the Mendelian hypothesis [IV vs VI, χ_2(3df)=4.16 p=0.24] and the hypothesis of no transmission was rejected [V vs VI, χ_2(2df)=8.2

p=0.01]. In summary, there was strong evidence for the presence of a major gene controlling caries, following a dominant model with an estimated frequency of the resistance allele "*A*" of 0.63.

DISCUSSION

The objective of this study was to conduct a CSA for dental decay in a collection of multiplex, multigenerational families recruited at the former leprosy Colony of Santo Antônio do Prata, located at the Amazonian state of Pará, north of Brazil. Environmental, socio-economical, educational and demographic variables are very homogenous throughout the colony (Lázaro *et al.*, 2010); in addition, characteristics such as a high cariogenic diet, low standards of oral hygiene and absence of fluoride in the water, known to have a major influence on caries development, make the Prata colony particularly suitable for genetic epidemiologic analysis of caries susceptibility.

The goal of CSA is to detect and discriminate between the different factors causing familial resemblance, ultimately aiming to demonstrate a major gene effect. Our CSA found the existence of a MG effect transmitted following a dominant model with the frequency of the resistance allele "*A*" estimated at 0.63. Mean DT was estimated at 1.53 for individuals with genotype AA and AB and 9.53 for BB individuals. The analysis was adjusted by age and gingivitis, both associated with the DT in our study population sample: DT increases with age only up to the 20-29 years old age class and decreases after that; patients with gingivitis have higher mean DT. From a clinical point of view, gingivitis is a result of plaque accumulation on the tooth surfaces owing to inadequate

oral hygiene. Gingivitis is a significant determinant of high incidence of caries and is compatible with the finding that plaque occurrence is also associated with dental decay (Julihn *et al.*, 2006). Number of teeth present in the mouth was not surprisingly associated with DT in the univariate analysis; however, the effect was totally explained by age, as demonstrated in the multivariate analysis.

Although CSA is ideal to identify the genetic model behind a specific phenotype, it has classic limitations. Different strategies of adjustment for non-familial variables may result in discordant findings across independent studies; our study population was heavily and homogeneously exposed to dental decay risk factors, minimizing the impact of non-familial variability and increasing the power of the analysis. Also, a more precise inference about the exact nature of the MG effect observed is impaired by the inability of the CSA to distinguish between one single, co-dominant or dominant gene with very strong effect or several co-dominant or dominant genes with milder effect that will play additively on the risk (Jarvik, 1998). For further investigation, molecular, DNA-based studies are necessary.

The results of the Prata Colony CSA on dental decay add to the evidence of a genetic component controlling the development of dental caries and lay ground for future genetic studies. The parameters generated in this CSA may be used for powerful, model-based linkage analysis followed by high-density association mapping using the already mapped Prata families, as used in previous studies (Mira *et al.*, 2004). To date, only one combined parametric and non-parametric linkage analysis has been conducted to detect genomic regions containing candidate genes for dental caries (Vieira *et al.*,

2008). Unfortunately, no data from the CSA performed by the authors was made available.

The description of the exact nature of the genetic component of the complex mechanism controlling susceptibility to human dental decay may ultimately lead to a better understanding of the physiopathological basis of this complex, chronic, multifactorial and commom disease and a Public Health concern, with potential impact over preventive strategies and, consequently, over public health systems worldwide.

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Table 1 - Demographic and clinical characteristics of the study population.

Demographic and Clinical Characteristics			DT	
Variable Group	Characteristic	Value	*p*-value Univariate	*p*-value Multivariate
Demographic	Ethnic group n (%)			
	Caucasian	26 (8.4)	0.32	
	Black	45 (14.6)		
	Mixed	237 (76.9)		
	Gender n (%)			
	Male	227 (50.3)	0.60	
	Female	224 (49.7)		
	Age mean±SD	30.72±16.31	0.005$^{\Psi}$	0.02$^{\Psi}$
Oral Health	Snack habit n (%)			
	Yes	216 (58.1)	0.03$^{\Psi}$	0.19
	No	156 (41.9)		
	Toothbrushing/day n (%)			
	Once	49 (13.2)	0.25	
	More than one time	321 (86.8)		
	Flossing every day n (%)			
	Yes	123 (33.2)	0.22	
	No	248 (66.8)		
	Dental appointment/year n (%)			
	None	227 (66.2)	0.07	
	One or more	116 (33.8)		
SES	Socioeconomic Status n (%)			
	No income	168 (56.8)	0.31	
	With income	128 (28.3)		
	Schooling n (%)			
	Up to primary school	125 (27.7)	0.95	
	Secondary school and plus	170 (37.6)		
Clinical	Gingivitis n (%)			
	Yes	146 (42.6)	0.0004$^{\Psi}$	0.0002$^{\Psi}$
	No	197 (57.4)		
	Plaque n (%)			
	Absent	96 (34.3)	0.08	
	Clinically visible	184 (65.7)		
	Number of teeth mean±SD	19.21±9.79	0.0001$^{\Psi}$	0.07
	Leprosy status n (%)			
	Yes	86 (23.1)	0.57	
	No	287 (76.9)		

$^{\Psi}$ Statistical significance. n = number of individuals with available information

Table 2 – Complex Segregation Analysis of quantitative phenotype DT, accounting for age and gingivitis status.

Model	Q_A	μ_{AA}	μ_{AB}	μ_{BB}	σ^2	ρSS	β rootage	β gengivitis	τAAB	τABB	τBBB	-2lnL+*C*
I. Sporadic	(0)	2.55	[=μ AA]	[=μ AA]	11.14	(0)	-0,08	1.52	-	-	-	108
II. FC												
SS	(0)	2.44	[=μ AA]	[=μ AA]	11.08	0.13	-0,06	1.51	-	-	-	104
III. MG and FC												
SS	0.63	1.49	[=μ AA]	9.44	3.87	0.10	-0,16	0.63	(1)	(0.5)	(0)	4
IV. MG	**0.63**	**1.53**	**[=μ AA]**	**9.53**	**3.88**	(0)	**-0,15**	**0.60**	(1)	(0.5)	(0)	4
V. Absence of Transmission	0.63	1.52	[=μ AA]	9.55	3.83	(0)	-0,18	0.59	0.63	0.63	0.63	8
VI. General Transmission	0.54	1.52	[=μ AA]	9.54	3.81	(0)	-0,18	0,62	1.00	0.52	0.41	0

Abbreviation: FC, familial correlation; SS, sibling-sibling; MG, major gene

"-" non-relevant parameter in the model

"( )" fixed parameter for hypothesis

"[ ]" parameter fixed to the same value as the preceding estimated parameter

"Q" frequency of leprosy predisposing allele "A"

"μ" genotypic mean for genotype (AA, AB, or BB), adjusted for covariate effects

"σ^{2}" Residual variance of the phenotype

"ρ" familial correlation

"β" covariable regression coefficients

"τ" τAAB, τABB, τBBB probabilities of transmitting "a" for individuals AA, AB, and BB, respectively

C = −1364.40, corresponding to twice the logarithm of the likelihood (2lnL) of the best-fitting model (model VI)

5. COMPLEMENTARY DISCUSSION

5.1 THE PRATA COLONY AS A MODEL TO STUDY DENTAL DECAY

One important aspect of this work is the nature of the population sample studied, recruited from the Vila do Santo Antonio do Prata (the Prata Colony), located 110 km east from Belém, the capital of the state of Pará (North of Brazil).

The Prata Colony was created by Franciscan monks in 1898 with the objective to evangelize the indians who were living on the site at that time. In early of 1920's, the place was chosen as an isolation site for leprosy patients, and the first leprosy affected individuals were transferred from the states of Pará and Maranhão. These early individuals constituted families and their descendents compose part of the population of around 2000 individuals living at the Prata Colony. Isolation was compulsory up to 1964; however, the Colony remains isolated until today, with very low migratory flux and very homogenous environmental, educational, SES, demographical and geographical variables, probably due to the strong stigma still associated with leprosy. Most of the families receive financial help from the government and only a few individuals have regular jobs. There is only one church and one school at the Colony, which are attended by all residents. In the same way, there is only one recreation park and one social club.

There is a public dental office at the Colony, but to obtain proper dental care is not straightforward. Moreover, the population is exposed to a highly cariogenic diet, associated with poor mouth hygiene habits and no access to treated and fluoridated water, as demonstrated by our data analysis. Despite these characteristics, epidemiological data for DMFT and its components obtained at the Prata Colony (Table 2) is not different for what was observed for the North region of Brazil, according to the latest Brazilian dental epidemiological survey (SB Brazil 2003).

Our hypothesis is that the homogeneity of non-genetic variables across the entire colony, together with poor oral health, makes the Prata Colony a unique, yet representative opportunity for genetic epidemiology studies on dental decay.

Table 2 – DMFT distribution by age class, Prata Colony and North of Brazil.

Age Class	Filled		Decayed		Missing		Sound		DMFT	
	Prata	North	Prata	North	Prata	North	Prata	North	Prata	North
12 years	0	0.45	2.14	2.27	0.35	0.36	23.92	22.77	2.5	3.13
15 to 19 years	0.45	1.20	2.22	3.43	1.33	1.34	23.81	22.56	4.04	6.14
35 to 44 years	0.31	1.89	2.34	2.97	13.44	14.77	11.90	11.54	16.09	19.88
65 to 74 years	0	0.17	0	1.77	25.77	26.38	2.22	3.34	25.77	28.34

Information Source of North Region - adapted from Costa *et al*., 2004. (Costa, Solla *et al.*, 2003)

5.2 LEPROSY AND MOUTH DISEASES

Previous studies have found positive evidence for association between leprosy and poor dental and periodontal health (Reichart, Ananatasan *et al.*, 1976; Costa, Nery *et al.*, 2003; Núnez-Marti, Bagán *et al.*, 2004; Pereira, Brito-de-Souza *et al.*, 2009). Authors speculate that the causes for such association are: (i) impaired oral hygiene resulting in plaque and calculus formation due to inability of leprosy patients to perform oral hygiene; (ii) mouth breathing, caused by specific granulomatous infiltrations of *Mycobacterium leprae* in the nasal cavity, and (iii) the anti-inflammatory effect of anti-leprosy drugs causing chronic gingival changes (Reichart, Ananatasan *et al.*, 1976).

All the components of the multi-drug therapy (MDT) recommended by WHO to treat leprosy (rifampicin, dapsone and clofazimine) cause a decrease in the production of saliva. This collateral effect may lead to increased colonization of the biofilm and impaired buffering capacity, ultimately resulting in an increase in caries, gingivitis and periodontitis occurrence and progression (Pedersen, Allan Bardow *et al.*, 2005; Dias, 2007).

5.3 DMFT INDEX LIMITATIONS

One of the most difficult problems to solve in research is the translation of clinical observations into measurable parameters suitable for

comprehensive interpretation and statistical analysis. In Dentistry, the DMFT index was proposed in the 1940's and since then, it has been broadly used as one of the simplest and most common used parameter in epidemiologic surveys of dental caries. The DMFT index quantifies dental health status based on the number of carious, missing and filled teeth. However, some limitations have been pointed out: (i) the index does not provide an accurate description of previous dental care (Anaise, 1984); (ii) the index value does not have a relation with the number of teeth at risk to develop dental decay (Antunes and Peres, 2006; Becker, Levin *et al.*, 2007); (iii) it does not provide information regarding the severity of the carious attack or the most adequate treatment (Anaise, 1984); (iv) in the past, dentists used to perform preventive fillings in teeth subjected to higher probability to develop the disease and/or with deep pit and fissure; these preventive fillings are a cause of overestimation of caries experience that is not accounted for in the DMFT (Antunes and Peres, 2006); (v) the DMFT index is merely the sum of three indicators of teeth health that may have different causes; for example, missing teeth can be due to caries and/or periodontal disease; (vi) the same weight is given for different components of the DMFT; for instance, a filled teeth without caries and a decayed teeth without treatment have the same impact over the index (Antunes and Peres, 2006); (vii) there is no distinction between different, sometimes extreme presentations for one DMFT component; for example, extensive carious destruction of the crown, indicative for root canal treatment is coded into the same category (decayed) as a simple carious lesion (Anaise, 1984), and (viii) the DMFT index does not consider health, but exclusively disease indicators; for example, healthy teeth are not considered in the index.

5.4 COVARIABLES NOT INCLUDED IN THE CSA

Several covariates included in this study were only significantly associated with DMFT and/or DT in the univariate analysis – significance was lost when they were included in the multivariate analysis, as described in table 1 of the manuscript (chapter 4). However, all these variables are known in the literature as having an important rule as a modifier factor for dental decay (Fejerskov and Manji, 1990) and will be discussed next.

In accordance with our study, when analyzing DMFT as the phenotype, snack habit has been demonstrated as an important influence in the development of the disease (Maciel, Marcenes *et al.*, 2001; Nobre dos Santos, Melo dos Santos *et al.*, 2002; Adair, Pine *et al.*, 2004). Dental decay incidence increases with the introduction of sugar in the diet. The deleterious effect of this dietary component is dependent on the ingested quantity and frequency, concentration in the food and type of ingested sugar (Dias, 2007).

Frequency of dental appointment is the result of a combination of several factors including, but not limited by, the professional counseling based on need for care, the patient's ability to pay and perceived value of oral health, as well as availability of providers (Manski, 2001). In the Prata families, a low frequency of dental appointment is associated with high DMFT, in accordance with several previous studies (Eckersley and Blinkhorn, 2001; Harris and Haycox, 2001).

In our study, lower individual SES was significantly related to DMFT in the univariate analysis. Individuals at the top of the socioeconomic ladder perform better in most health parameters (World Health Organization, 2004), indicating that it is impossible to disconnect living conditions of a population with changes in the disease-health process. Lower SES level has been strongly related to lower education (Comissão Nacional sobre Determinantes Sociais da Saúde, 2008), lower motivation and more caries (Dias, 2007). Lower social class has been also significantly associated with high DMFT (Pine, 1997; Patussi, Hardy *et al.*, 2006). In a community such as the Prata Colony, where individuals do not receive proper assistance regarding habitation, alimentation, general health, sanitation, education and culture, it seems to be difficult to have motivation towards good oral hygiene.

After multivariate analysis of the Prata pedigrees, the only variables kept in the DMFT model were age and leprosy, and age and gingivitis for DT, demonstrating that these variables are sufficient to explain all the other determinant and modifier factors for dental decay observed in the univariate analysis (DMFT or DT). This effect may be due to the homogenous exposure of the population to these caries-associated, non-genetic variables.

5.5 LIMITATIONS OF THE STUDY

5.5.1 Sample Size Calculation

There is no reliable method to calculate the sample size required for a desired level of power to detect a Mendelian locus by CSA. Sample size for CSA is usually determined by the characteristics of the study phenotype and range from hundreds – for common traits – to thousands individuals when rare, Mendelian disorders are studied. Of note, a smaller number of large families is preferred over a large number of small families (reducing the need for a large sample size) as they result on an increase in transmission information (Jarvik, 1998).

5.5.2 Interviewer Bias

The social attitude of the interviewer may influence and bias the interview. For example, an interviewer may support or oppose to a particular cause under investigation, or feel uncomfortable when asking sensitive questions. Voice tone, mannerism and body language presented by the interviewer may influence the way a subject reacts to a question (Cavazara, 2003). In addition, the expectations of the interviewer towards the analytic goals may influence how he/she performs, hear, and record. For example, when facing an obscure or confusing reply, an interviewer may mark the answers that he/she thinks may be correct. Selective or partial recording is also another source of interviewer bias, mainly when working with open-ended questions (Aday, 1996; Cavazara, 2003).

In this study, the researcher that performed most of the work, in all stages, was also the solely interviewer. If from one perspective, having one single interviewer may be positive – for reducing variability of the data collection process – it is also possible that, in the absence of inter-examiner error checking, this may have been a source of bias. To decrease this type of bias, clinical examination of the research subjects was performed after the interview, therefore applied by an interviewer with no previous knowledge of the mouth health status of the subject.

5.5.3 Diagnostic Bias

As discussed before, phenotype characterization is crucial for the success of genetic analysis. In our study, all clinical examination was conducted in the field, far from the ideal set up of a well-equipped dental office and resources such as visual-tactile and radiographic exam, translumination with optical fiber and electric resistance tests (Fejerskov, 2004; Departament of Health and Human Services, 2005). To minimize error, for clinical assessment a simplified criterion for diagnosis and coding protocol established by WHO, and worldwide employed, was applied (WHO, 1987).

5.6 PUBLIC POLICIES AND GENETICS

The severity of genetic diseases is variable: some pathologies are lethal to the embryo and lead to spontaneous abortion in 12 to 15% of associated pregnancies (Curry, 1992); other diseases can have a profound impact over the development and quality of life of the individual, giving rise to deficiencies, incapacities and chronic morbidities (Wilkinson and Marmot, 2003; World Health Organization, 2004). In Brazil, assistance for monogenic and chromosomal diseases was recently implemented as part of the services offered by the Unified Health Services (*Serviço Único de Saúde* – SUS, the Brazilian public health system, in portuguese) and genetic clinics became eligible to receive governmental accreditation (Federal Governmental Decree # 81, 20th of January, 2009). Therefore, therapeutic and prophylactic treatment are available in Brazil for both individuals affected and at risk to develop the disease, e.g., individuals harboring the causative genetic mutation. However, for multifactorial genetic diseases of complex etiology, preventive treatments are not included. As basic research is moving towards a better knowledge of the molecular basis of common, complex diseases such as dental decay, a deeper discussion of the impact of these new, exciting advances over the public health systems worldwide, and as a consequence, over the quality of life of large populations, is becoming increasingly necessary.

5.7 FINAL REMARKS AND FUTURE PERSPECTIVES

Here we present the results of the first CSA performed for dental decay, using a model population consisting of characteristics that increase the power of the analysis to detect the existence of genetic factors controlling the phenotype. The natural next step is to use the information produced here in powerful, gene mapping studies, such as parametric linkage analysis, aiming to the complete dissection of the genetic component controlling human susceptibility to dental decay. Ultimately, knowing *a priori* whose individuals have more chance to develop caries will allow dentists to increase their focus on preventive and health promotion techniques to be offered to this particularly susceptible sub-population.

6. CONCUSIONS

1 The Prata population sample included in this study presented a male/female ratio of 1.01 and a mean age at enrollment of 30.72 years old. The means for DMFT, decayed teeth, missing teeth and filled teeth were 10.99, 2.44, 8.20 and 0.33, respectively;

2 Univariate analysis revealed that the variables age, snack habit, frequency of dental appointments, socioeconomic status, schooling, gingivitis and leprosy were associated with DMFT in the study population. After multivariate analysis, only age and leprosy remained associated;

3 Univariate analysis revealed that the variables age, snack habit, gingivitis and number teeth were associated with DT in the study population. After multivariate analysis, only age and gingivitis remained associated;

4 A major gene effect controlling the DMFT index was detected, with the best fit genetic model being co-dominant; the frequency of the resistance allele *A* was 0.52 and the mean DMFT was estimated as 1.83, 3.07 and 3.78 for AA, AB and BB individuals, respectively;

5 The major gene effect detected for DMFT followed a Mendelian transmission pattern and was enough to explain the familial aggregation observed;

6 A major gene effect controlling DT was detected, with the best fit genetic model being dominant; the frequency of the resistance allele *A* was 0.63 and the mean DT was estimated as 1.53 for individuals with genotype AA and AB and 9.53 for BB individuals;

7 The major gene effect detected for DT followed a Mendelian transmission pattern and was enough to explain the familial aggregation observed.

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APPENDICES

APPENDIX A: RATIONALE AND HYPOTHESIS, OBJECTIVES AND CONCLUSIONS-PORTUGUESE

JUSTIFICATIVA E HIPÓTESE

A cárie dentária é uma doença complexa, crônica, multifatorial e ainda uma das doenças mais comuns na Odontologia e nas populações, sendo seu conhecimento de grande importância para a Saúde Pública. Fatores não-genéticos (determinantes e modificadores) têm sido exaustivamente discutidos na literatura e tratamentos preventivos têm sido desenvolvidos, baseados nos achados destes estudos. Entretanto, estudos recentes têm identificado a existência de fatores genéticos influenciando na susceptibilidade da cárie dentária nas populações humanas. Embora os achados sejam significantes, estes estudos genéticos falham em descrever o modelo genético de herança envolvido (dominante/co-dominante/recessivo), freqüências alélicas, médias e variâncias dos genótipos, como pode ser obtido ao conduzirmos uma análise de segregação complexa (ASC). Os parâmetros estimados pela ASC podem ser de subseqüente uso em estudos de mapeamento genético. Nossa hipótese é que existe um efeito genético controlando a susceptibilidade do hospedeiro à cárie dentária, forte o suficiente para ser detectada por uma ASC.

OBJETIVOS

OBJETIVO GERAL

Descrever o modelo de herança genético que melhor explica o padrão de segregação observado para a cárie dentária numa amostra de famílias multigeracionais de uma população isolada no Norte do Brasil.

OBJETIVOS ESPECÍFICOS

I. Descrever as variáveis clínicas e epidemiológicas relacionadas com a cárie nesta população de estudo;
II. Testar as variáveis clinicas e epidemiologicas para associação com cárie na amostra populacional;
III. Investigar a existencia de um efeito genetic controlando os fenótipos CPOD e dentes cariados (CD).

CONCLUSÕES

1 A amostra populacional deste estudo possui uma razão homem/mulher de 1,01 e idade média no recrutamento de 30,72 anos. As médias do CPOD, dentes cariados, perdidos e obturados foram de 10,99, 2,44, 8,20 and 0,33, respectivamente;

2 As análises univariadas revelaram que as variáveis idade, hábito de lanches entre refeições, freqüência de visitas ao dentista, status socioeconômico, escolaridade, gengivite e hanseníase estavam associadas com CPOD na população estudada. Depois de conduzida a análise multivariada, apenas idade e hanseníase permaneceram associadas com CPOD;

3 As análises univariadas revelaram que as variáveis idade, hábito de lanches entre refeições, gengivite e número de dentes presentes estavam associadas com CD na população estudada. Depois de conduzida a análise multivariada, apenas idade e gengivite permaneceram associadas com CD;

4 Um efeito genético principal controlando o fenótipo CPOD foi detectado, sendo que o melhor modelo possui um efeito co-dominante, freqüência do alelo de resistência *A* de 0,52 e média do CPOD estimado em 1,83, 3,07 e 3,78 para os genótipos AA, AB e BB, respectivamente;

5 O efeito genético principal detectado para o fenótipo CPOD segue o padrão de transmissão Mendeliano e foi suficiente para explicar a agregação familiar observada;

6 Um efeito genético principal controlando o fenótipo CD foi detectado, sendo que o melhor modelo possui um efeito dominante, freqüência do alelo de resistência *A* de 0,63 e média do CD estimado em 1,53, para indivíduos com genéotipo AA e AB e 9,53 para os indivíduos BB;

7 O efeito genético principal detectado para o fenótipo CD segue o padrão de transmissão Mendeliano e foi suficiente para explicar a agregação familiar observada.

APPENDIX B: CHART FOR DENTAL EXAMINATION AND QUESTIONNAIRE – PORTUGUESE

Estudo Genético Epidemiológico de

Susceptibilidade à Cárie Dentária

Cód da família:	**Cód do voluntário:**

Data:	Duplicata INTRA:
Nome:	
Local de nascimento:	Sexo: Feminino □ Masculino□
Data de nascimento:	Idade:
Setor da Colônia: 1 2 3 4 5 6	
Grupo Étnico (B, N, M, A, I):	
Hanseníase: Sim□ Não□	
Quanto tempo mora na Colônia:	
Qual parente primeiro imigrou para Colônia:	

16V 12V 24P

□ □ □

□ □ □

IP modificado - Características:

0 – **ausência** de placa
1 - placa **clinicamente visível**
2 – placa **abundante**

44V 32L 36L

CPO-D

17	16	15	14	13	12	11	21	22	23	24	25	26	27
□	□	□	□	□	□	□	□	□	□	□	□	□	□

47	46	45	44	43	42	41	31	32	33	34	35	36	37
□	□	□	□	□	□	□	□	□	□	□	□	□	□

CPO-D:

Observações durante exame:_____

_____

GENGIVITE

( ) SIM ( ) NÃO

PERIODONTITE

( ) SIM ( ) NÃO

Códigos Diagnóstico:

0 – Hígido
1- Cárie ativa
2- Restaurado, com cárie ativa
3- Restaurado, sem cárie
4- Ausente devido à cárie
5- Ausente por outra razão
6- Fluorose
7- Erosão
8- Selado
9- Suporte para prótese, coroa protética ou faceta / implante
10- Dente não irrompido
11- Dente excluído
T – Traumatismo

Questionário do Dentista

Quantos membros de sua família moram com você?

(A) Nenhum.
(B) Um ou dois.
(C) Três ou quatro.
(D) Cinco ou seis.
(E) Mais do que seis.

Quem são eles?

Nome	Parentesco	Código
1-		
2-		
3-		
4-		
5-		
6-		
7-		
8-		
9-		
10-		

Relate o que você comeu ontem:

Manhã	**Entre manhã e tarde**	**Almoço**	**Entre almoço e Jantar**	**Jantar**

Porções:
Carboidratos:_____
Proteínas:_____
Carnes: _____
Derivados de leite: _____
Vegetais e frutas:
Graus e derivados: _____(pão, cereal, massa, arroz, etc)

Quantas vezes você ingere açúcar (salgadinhos, bolachas, lanchinhos), por dia, fora das refeições?
nenhuma vez ( ) até 3 vezes ( ) mais de 3 vezes ( )

Você escovou seus dentes hoje?
sim ( ) não ( )
Quantas vezes você escova os dentes por dia?
nenhuma vez ( ) uma vez ( )
mais de uma vez ( )
Você usa fio dental?
sim ( ) não ( )
Caso sua resposta anterior tenha sido sim, relate quando você usa fio dental:
todos os dias ( ) de vez em quando

Toma algum remédio?
sim ( ) não ( )
Qual?_____
Você tomou antibiótico nos últimos três meses?
sim ( ) **não ( )**

Já foi ao dentista?
Sim ( ) Não ( )
Quantas vezes ao ano vai ao dentista?
nenhuma vez ( ) 1 vez ( )
2 vezes ou mais ( )
Quem na sua família já foi ao dentista?
1 Ninguém,
2 Mãe,
3 Pai,
4 Algum dos seus filhos,
5 Irmão,
6 Marido/Esposa,
7 Outro (por favor, especifique)_____
Alguma vez já recebeu informações sobre a prevenção de cáries e doenças bucais?
1 Não
2 Sim
3- Não sei

Usa flúor?
sim ( ) não ( )
Caso sua resposta anterior tenha sido sim, relate quando você utiliza flúor:
todos os dias ( ) de vez em quando ( ) só no

Autopercepção em Saúde Bucal

Como classifica sua saúde bucal?	0-Não sabe/ Não informou	1-Péssima	2-Ruim	3-Regular	4-Boa	5-Ótima
Como classificaria a aparência de seus dentes?	0- Não sabe/ Não informou	1-Péssima	2- Ruim	3-Regular	4-Boa	5-Ótima
Como classificaria sua mastigação?	0- Não sabe/ Não informou	1-Péssima	2- Ruim	3-Regular	4-Boa	5-Ótima
Como classificaria a sua fala devido aos seus dentes e gengivas?	0- Não sabe/ Não informou	1-Péssima	2- Ruim	3-Regular	4-Boa	5-Ótima
De que forma a sua saúde bucal afeta o seu relacionamento com outras pessoas?	0-Não sabe/ Não informou	1-Péssima	2- Ruim	3-Regular	4-Boa	5-Ótima
O quanto de dor seus dentes e gengivas causaram nos últimos 6 meses?	0-Nenhuma dor	1-Pouca dor	2-Média dor	3-Muita dor	XXXX	XXXXX

OBS: Famílias com ligação a esta:

_____
_____
_____
_____
_____
_____
_____

APPENDIX C: CHART FOR DENTAL EXAMINATION AND QUESTIONNAIRE – ENGLISH

Genetic Epidemiologic Study of the Dental Decay Susceptibility

Family Code:	**Individual Code:**

Date:	Duplication INTRA:
Name:	
Local de nascimento:	Gender: Female □ Male□
Date of brith:	Age:
Sector in the Colony: 1 2 3 4 5 6	
Etnic Group (B, N, M, A, I):	
Leprosy: Yes□ No□	
How long do you live in the Colony?	
Who was the first parent that came to the Colony?	

16V 12V 24P

□ □ □

□ □ □

44V 32L 36L

IP modified - Characteristics:

0 – no plaque
1 – plaque clinically visible
2 – abundant plaque

DMFT

17	16	15	14	13	12	11	21	22	23	24	25	26	27
□	□	□	□	□	□	□	□	□	□	□	□	□	□
47	46	45	44	43	42	41	31	32	33	34	35	36	37
□	□	□	□	□	□	□	□	□	□	□	□	□	□

CPO-D:

Observation during the exam:_____

_____

GINGIVITI

( ) SIM ( ) NÃO

PERIODONTITIS

( ) SIM ( ) NÃO

Diagnostic Codes:

0 – Healthy
12- Active decay
13- Restored, with active decay
14- Restored, without active decay
15- Absent due to decay
16- Absent due to other raison
17- Fluorosis
18- Tooth erosion
19- Tooth sealed
20- Support for prosthesis, crown prosthodontics or facet or implant
21- Not erupted tooth
22- Excluded tooth
T – Traumatism

Questionnaire

How many members of your family live with you?

(A) None.
(B) One or two.
(C) Three or four.
(D) Five or six.
(E) More than six.

Who are they?

Name	Relationship	Code
1-		
2-		
3-		
4-		
5-		
6-		
7-		
8-		
9-		
10-		

What did you eat yesterday?

Breakfast	**Between breakfast and lunch**	**Lunch**	**Between lunch and dinner**	**Dinner**

Portion:
Carbohydrate:_____
Proteins:_____
Meat: _____
Dairy Products: _____
Vegetables and fruits:
Grain and derivates:_____(bread, cereal, pasta, rice, etc)

How many times you consume sugar during the day, except meals?
never ( ) until 3 times ( ) more than 3 times ( )

Did you brush your teeth today?
yes ( ) no ( )
How many times do you brush your teeth per day?
never ( ) once ( )
more than once ( )
Do you use dental floss?
yes ( ) no ( )
If yes, when do you use it?

Do you take any medication?
yes ( ) no ( )
Which one?_____
Did you take antibiotic in the last three months?
yes ( ) no ( )

Have you ever gone to the dentist?
yes ( ) no ( )
How many times?
never ( ) once ( ) twice or more ( )
Who in your family went to the dentist?
1 no one,
2 mother,
3 father,
4 my son/daugther,
8 brother,
9 husband/wife,
10 other (please, especify)_____
Have you ever received any information about dental decay and mouth disease prevention?
1 no
2 yes
3- I don't know

Do you use fluoride?
yes ( ) no ( )
If yes, when do you use fluoride?
everyday ( ) sometimes ( ) only when I go to the

Oral health self perception

How do you classify your oral health?	0- I don't know/ Did not inform	1-Terrible	2-Bad	3-Regular	4-Good	5-Excelent
How would you classify the appearance of your teeth?	0- I don't know/ Did not inform	1-Terrible	2-Bad	3-Regular	4-Good	5-Excelent
How would you classify your crunch?	0- I don't know/ Did not inform	1-Terrible	2-Bad	3-Regular	4-Good	5-Excelent
How would you classify your talking due to your teeth and gum?	0- I don't know/ Did not inform	1-Terrible	2-Bad	3-Regular	4-Good	5-Excelent
How your oral health affect your relationship with other people?	0- I don't know/ Did not inform	1-Terrible	2-Bad	3-Regular	4-Good	5-Excelent
How much pain your teeth and gum caused to you in the last six months?	0-None	1-A few	2-Medium	3-A lot of pain	XXXX	XXXX

OBS: Linkage families:

_____
_____
_____
_____
_____
_____
_____

APPENDIX D: INFORMED CONSENT FOR INDIVIDUALS UNDER 18 YEARS OLD - PORTUGUESE

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Estudo Genético Epidemiológico de Susceptibilidade à Cárie Dentária

A Pontifícia Universidade Católica do Paraná (PUCPR) estará realizando um estudo que irá investigar o papel da genética na susceptibilidade à cárie e na gravidade da doença. Se bem sucedido, o projeto poderá facilitar o tratamento da cárie e melhorar a qualidade de vida dos pacientes. Este estudo será coordenado por Dr. Marcelo Távora Mira, professor adjunto e pesquisador do programa de Pós-Graduação em Ciências da Saúde da PUCPR e realizado pela Dra Renata Iani Werneck, dentista e doutoranda do Pós-Graduação em Ciências da Saúde da PUCPR.

1) Métodos

Se você concordar em participar neste estudo, ele/ela será submetido(a) a um exame clínico odontológico e a um questionário. O exame será constituído de uma observação dos dentes do(a) seu(sua) filho(a) e gengiva. A dentista irá usar luvas, um palito de madeira para afastar a bochecha e gaze para absorver saliva. Os dados observados serão anotados em um folha (odontograma) e serão analisados e guardados pelos pesquisadores na PUCPR. Estes dados irão permitir aos cientistas estudar as características genéticas que tornam mais fácil para alguns indivíduos e seus familiares desenvolver a cárie. Sob sua autorização, informações de prontuários clínicos também poderão ser lidas pelos cientistas e utilizadas no estudo.

Sua autorização só valerá sob a condição de se manter o desenho, objetivos e metodologia do projeto original, além da avaliação e aprovação de eventuais alterações pelo Comitê de Ética da PUCPR.

2) Riscos Físicos para Saúde/Desconfortos

Os riscos físicos para saúde de participação neste estudo são muito pequenos e limitados ao procedimento de exame clínico. Durante o exame, você poderá sentir um desconforto temporário devido o uso do palito de madeira.

3) Alternativas

Se seu (sua) dependente tiver cárie, uma visita ao dentista será indicada, mesmo que você não queira participar do estudo. Portanto, se seu (sua) dependente decidir não autorizar, ou cessar a participação de seu dependente no estudo a qualquer momento, seu dependente receberá informações sobre a saúde da boca dele, assim como explicações sobre prevenção de doenças da boca.

Se seu dependente não tiver cárie, você está sendo convidado a autorizar a participação dele/dela no estudo como familiar do afetado. Neste caso, sua decisão

de autorizar ou não a participação dele/dela, ou de cessar sua participação a qualquer momento, não irá interferir de nenhuma forma nos procedimentos odontológicos para diagnóstico ou tratamento da cárie que você possa necessitar no futuro. Da mesma forma, sua decisão não irá refletir no acesso a procedimentos odontológicos necessários a algum familiar.

4) Custos para os participantes

No caso de você decidir autorizar a participação de seu (sua) dependente no estudo, você não terá nenhum custo. Todos os custos serão cobertos pelo estudo.

5) Benefícios

Em longo prazo, os resultados deste estudo poderão facilitar a detecção de causas da cárie ocorrer, tornando possível evitar o desenvolvimento da doença. Além disso, espera-se que conhecimentos científicos adicionais sejam alcançados, com conseqüente melhoria do tratamento de pessoas afetadas pela cárie.

6) Reembolso

Nem você nem seu dependente serão reembolsado por participar deste estudo.

7) Confidencialidade dos dados

A participação em projetos de pesquisa pode resultar em perda de privacidade. Além disso, a descoberta de fatores de risco genéticos para cárie podem expor susceptibilidades de certos grupos de pessoas, possivelmente levando a outros ou certas empresas a considerar estes grupos diferentes de uma forma negativa. Entretanto, procedimentos serão adotados pelos responsáveis por este estudo no intuito de proteger a confidencialidade das informações que você fornecer e as informações produzidas pelo projeto. Nenhuma informação genética e odontológica individual será tornada pública. As informações serão codificadas e mantidas num local reservado o tempo todo. Somente os pesquisadores envolvidos neste estudo terão acesso às informações. Após o término deste estudo, as informações serão transcritas dos questionários para arquivos de computador, mantidos em local restrito com acesso permitido apenas aos mesmos pesquisadores. Os dados deste estudo poderão ser discutidos com pesquisadores de outras instituições, mas nenhuma identificação será fornecida.

8) Autorização para utilização de dados coletados na pesquisa "Estudo de fatores de risco moleculares de susceptibilidade do hospedeiro à hanseníase"

Caso seu (sua) dependente já tenha sido entrevistado para o estudo sobre hanseníase, também realizado por nosso grupo e chamado "Estudo de fatores de risco moleculares de susceptibilidade do hospedeiro à hanseníase", ao assinar este Termo de Consentimento Livre e Esclarecido você também estará autorizando o uso das informações já coletados sobre ele/ ela e sua família, agora para estudar cárie. Esta autorização evitará que os pesquisadores tenham que fazer novamente uma

série de perguntas para seu (sua) dependente que ele/ela já respondeu, tornando a pesquisa mais eficiente.

CONSENTIMENTO

Estudo Genético Epidemiológico de Susceptibilidade à Cárie Dentária

Você receberá uma copia deste consentimento para mantê-lo consigo. Nos próximos dias, se tiver qualquer duvida sobre a participação de seu dependente neste estudo, poderá utilizar os seguintes meios de contato com o pesquisador responsável:

Número Prontuário:_____ Número da Ficha:_____

Marcelo Távora Mira
Telefone: (41) 3271-2618
Celular: (41) 9164-4045
E-mail: m.mira@pucpr.br

A PARTICIPAÇAO EM PESQUISA É VOLUNTARIA

Você tem o direito de não autorizar a participação de seu dependente, ou mesmo de cessar a participação de seu dependente do estudo em qualquer momento que queira, sem riscos para o tratamento odontológico dele/dela. Se você autoriza a participação do seu dependente, você deve assinar na linha abaixo.

Se você autoriza a participação de seu dependente no estudo, permitirá que seu endereço e telefone sejam anotados em uma folha separada, para facilitar contato quando necessário. Como já foi esclarecida anteriormente, toda informação, pessoal será mantida em sigilo.

_____ _____

Nome completo do dependente Número do prontuário

_____ _____

Assinatura do pai ou responsável legal — Nome completo do pai/resp. legal

_____ _____

Assinatura do entrevistador — Nome do entrevistador

_____ _____

Assinatura testemunha 1 — Assinatura testemunha 2

___/___/___
Data

APPENDIX E: INFORMED CONSENT - PORTUGUESE

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
Estudo Genético Epidemiológico de Susceptibilidade à Cárie Dentária

A Pontifícia Universidade Católica do Paraná (PUCPR) estará realizando um estudo que irá investigar o papel da genética na susceptibilidade à cárie e na gravidade da doença. Se bem sucedido, o projeto poderá facilitar o tratamento da cárie e melhorar a qualidade de vida dos pacientes. Este estudo será coordenado por Dr. Marcelo Távora Mira, professor adjunto e pesquisador do programa de Pós-Graduação em Ciências da Saúde da PUCPR e realizado pela Dra Renata Iani Werneck, dentista e doutoranda do Pós-Graduação em Ciências da Saúde da PUCPR.

1) Métodos

Se você concordar em participar neste estudo, você será submetido a um exame clínico odontológico e a um questionário. O exame será constituído de uma observação dos seus dentes e gengiva. A dentista irá usar luvas, um palito de madeira para afastar a bochecha e gaze para absorver saliva. Os dados observados serão anotados em um folha (odontograma) e serão analisados e guardados pelos pesquisadores na PUCPR. Estes dados irão permitir aos cientistas estudar as características genéticas que tornam mais fácil para alguns indivíduos e seus familiares desenvolver a cárie. Sob sua autorização, informações de prontuários clínicos também poderão ser lidas pelos cientistas e utilizadas no estudo.
Sua autorização só valerá sob a condição de se manter o desenho, objetivos e metodologia do projeto original, além da avaliação e aprovação de eventuais alterações pelo Comitê de Ética da PUCPR.

2) Riscos Físicos para Saúde/Desconfortos

Os riscos físicos para saúde de participação neste estudo são muito pequenos e limitados ao procedimento de exame clínico . Durante o exame, você poderá sentir um desconforto temporário devido o uso do palito de madeira.

3) Alternativas

Se você tiver cárie, uma visita ao dentista será indicada, mesmo que você não queira participar do estudo. Portanto, se você decidir não participar, ou cessar sua participação no estudo a qualquer momento, você receberá informações sobre a saúde da sua boca, assim como explicações sobre prevenção de doenças da boca.
Se você não tiver cárie, você está sendo convidado a participar do estudo como familiar do afetado. Neste caso, sua decisão de participar ou não, ou de cessar sua participação a qualquer momento, não irá interferir de nenhuma forma nos

procedimentos odontológicos para diagnóstico ou tratamento da cárie que você possa necessitar no futuro. Da mesma forma, sua decisão não irá refletir no acesso a procedimentos odontológicos necessários a algum familiar.

4) Custos para os participantes

No caso de você decidir participar do estudo, você não terá nenhum custo. Todos os custos serão cobertos pelo estudo.

5) Benefícios

Em longo prazo, os resultados deste estudo poderão facilitar a detecção de causas da cárie ocorrer, tornando possível evitar o desenvolvimento da doença. Além disso, espera-se que conhecimentos científicos adicionais sejam alcançados, com conseqüente melhoria do tratamento de pessoas afetadas pela cárie.

6) Reembolso

Você não será reembolsado por participar deste estudo.

7) Confidencialidade dos dados

A participação em projetos de pesquisa pode resultar em perda de privacidade. Além disso, a descoberta de fatores de risco genéticos para cárie podem expor susceptibilidades de certos grupos de pessoas, possivelmente levando a outros ou certas empresas a considerar estes grupos diferentes de uma forma negativa. Entretanto, procedimentos serão adotados pelos responsáveis por este estudo no intuito de proteger a confidencialidade das informações que você fornecer e as informações produzidas pelo projeto. Nenhuma informação genética e odontológica individual será tornada pública. As informações serão codificadas e mantidas num local reservado o tempo todo. Somente os pesquisadores envolvidos neste estudo terão acesso às informações. Após o término deste estudo, as informações serão transcritas dos questionários para arquivos de computador, mantidos em local restrito com acesso permitido apenas aos mesmos pesquisadores. Os dados deste estudo poderão ser discutidos com pesquisadores de outras instituições, mas nenhuma identificação será fornecida.

8) Autorização para utilização de dados coletados na pesquisa "Estudo de fatores de risco moleculares de susceptibilidade do hospedeiro à hanseníase"

Caso você já tenha sido entrevistado para o estudo sobre hanseníase, também realizado por nosso grupo e chamado "Estudo de fatores de risco moleculares de susceptibilidade do hospedeiro à hanseníase", ao assinar este Termo de Consentimento Livre e Esclarecido você também estará autorizando o uso das informações já coletados sobre você e sua família, agora para estudar cárie. Esta autorização evitará que os pesquisadores tenham que lhe fazer novamente uma série de perguntas que você já respondeu, tornando a pesquisa mais eficiente.

CONSENTIMENTO

Estudo Genético Epidemiológico de Susceptibilidade à Cárie Dentária

Você receberá uma cópia deste Termo de Consentimento para mantê-lo consigo. Se você tiver qualquer duvida no futuro sobre a sua participação neste estudo, você pode e deve utilizar os seguintes meios de contato com o pesquisador responsável:

Dr. Marcelo Távora Mira
Telefone: (41) 3271-2618
Celular: (41) 9164-4045
E-mail: m.mira@pucpr.br

A PARTICIPAÇAO EM PESQUISA É VOLUNTARIA

Você tem o direito de não concordar em participar ou mesmo de se retirar do estudo em qualquer momento que queira, sem riscos para o seu tratamento odontológico. Se você desejar e concordar em participar, deve assinar ou fornecer sua impressão digital na linha apropriada abaixo.

Se você desejar participar do estudo, permitirá que seu endereço e telefone sejam anotados em uma folha separada, para facilitar contato quando necessário. Como já foi esclarecida anteriormente, toda informação, pessoal será mantida em sigilo.

_____ _____
Assinatura ou impressão digital do voluntário Nome completo e nº do prontuário

_____ _____
Assinatura do entrevistador Nome do entrevistador

_____ _____
Assinatura testemunha 1 Assinatura testemunha 2

_____/_____/_____
Data

APPENDIX F: INFORMED CONSENT FOR INDIVIDUALS UNDER 18 YEARS OLD - ENGLISH

INFORMED CONSET

A Genetic Epidemiologic Study of Dental Decay Susceptibility

The Pontifical Catholic University of Paraná (PUCPR) will be conducting a study that will investigate the role of genetics on susceptibility to dental decay and severity of disease. If successful, the project may facilitate treatment of leprosy and improve the quality of life of the patients, their contacts and relatives. This study will be coordinated by Dr. Marcelo Távora Mira, adjunct professor and researcher of the Post-Graduation Program in Health Sciences of PUCPR and conducted by Dra. Renata Iani Werneck, dentist and PhD student of the Post-Graduation Program in Health Sciences of PUCPR.

1) Methods

If you authorize your son/daughter or legal dependent to participate in this study, you will be submitted to a mouth clinical examination and to a questionnaire. The examination will be the observation of your son/daughter teeth, mouth and gum. The dentist will use gloves, natural light, tongue depressor and gauze. The data will be wrote down and will be analyzed by the researchers at PUCPR. These data will permit the scientists to study the genetic characteristics of dental decay. Upon your authorization, information from your clinical records will be evaluated by the scientists and used in the study.

2) Risk/Side Effects

The physical health risks of participating in this study are very small and limited to the clinical examination. During the examination, your dependent may feel temporary discomfort due to the use of the tongue depressor.

3) Alternatives

If your son/daughter is affected by dental decay, a visit to a dentist will be indicated, whether or not you decide to participate in the study. Therefore, if your son/daughter decides not to participate, or to cease your participation in the study at any moment, all procedures for diagnosis as also information about prevention will be given.

If your son/daughter is not affected by dental decay, he/she is being asked to participate in the study as part of the relative of an affected individual. In this case, your decision whether or not to participate, or to cease his/her participation at any moment, will not interfere in any way with medical procedures for diagnosis or treatment of dental decay you may need in the future. Likewise, your decision will not reflect on dental procedures needed by an affected relative or contact you may have.

4) Cost for the participant

In case you decide to authorize your dependent to participate in the study, there will be no costs for you. All costs will be covered by the study.

5) Benefits

In the long term, the results of this study, to which your dependent will be submitted, may facilitate the detection of dental decay causes, making it possible to avoid inadequate treatments. In addition, it is expected that additional scientific knowledge will be achieved, with consequent improvement of treatment of people affected by dental decay.

6) Reimbursement

You or your dependent will not be reimbursed for participating in this study

7) Confidentiality of the data

The participation in research projects may result in loss of privacy. In addition, discovery of genetic risk factors for dental decay may expose susceptibilities of certain groups of people, possibly inducing others or certain companies to consider these groups different in a negative way. However, procedures will be adopted by the supervisors of the study in order to protect the confidentiality of the information your dependent provide and the information produced by the project. No individual genetic information will be made public. All information will be coded and kept in a place with restricted access during all times. Only the researchers involved in the study will have access to the information. After the end of the study the information will be transcribed to computer files and will be kept in a place with access restricted to the researchers involved. The data obtained from this study may be discussed with researchers from other institutions, but no identification will be provided.

8) Authorization for the use of the data collected in the study "A study of host genetic risk factors for leprosy susceptibility"

In case your dependent has already been interviewed for the leprosy study, also conducted by our study group and named "A study of host genetic risk factors for leprosy susceptibility", if you sign this informed term you will also authorize us to use the data collected about your dependent and your family in the dental decay study. This authorization will avoid that the researchers make again the same questions, which were asked before.

TERM OF CONSENT

A Genetic Epidemiologic Study of Dental Decay Susceptibility

You will receive and keep a copy of this term of consent. If you have any questions in the future about the participation of your dependent in the study, please don't hesitate to use the following phone numbers and e-mail address to contact the principal investigator of the study:

Prontuary number:_____ File number:_____

Marcelo Távora Mira
Phone number: (41) 3271 2619
Cell phone number: (41) 9164 4045
E-mail address: m.mira@pucpr.br

PARTICIPATION IN THE STUDY IS VOLUNTARY

You have the right not to authorize the participation of your dependent in the study, or even cease his/her participation at any moment you want, with no risk for his/her medical treatment. If you authorize his/her participation, Iyou shall sign or provide your digital print on the line below.

If you authorize the participation of your dependent, you will permit that your address and your phone number be registered on a separate form, in order to facilitate contact when necessary. As discussed before, all the information will be kept confidential.

_____ _____

Full name of the dependent | Prontuary number

_____ _____

Signature of the father or legal guardian | Father or legal guardian full name

_____ _____

Signature of Interviewer | Name of Interviewer

_____ _____

Signature of Witness 1 | Signature of Witness 2

___/___/___

Date

APPENDIX G: INFORMED CONSENT - ENGLISH

INFORMED CONSET
A Genetic Epidemiologic Study of Dental Decay Susceptibility

The Pontifical Catholic University of Paraná (PUCPR) will be conducting a study that will investigate the role of genetics on susceptibility to dental decay and severity of disease. If successful, the project may facilitate treatment of leprosy and improve the quality of life of the patients, their contacts and relatives. This study will be coordinated by Dr. Marcelo Távora Mira, adjunct professor and researcher of the Post-Graduation Program in Health Sciences of PUCPR and conducted by Dra. Renata Iani Werneck, dentist and PhD student of the Post-Graduation Program in Health Sciences of PUCPR.

1) Methods

If you agree to participate in this study, you will be submitted to a mouth clinical examination and to a questionnaire. The examination will be the observation of your teeth, mouth and gum. The dentist will use gloves, natural light, tongue depressor and gauze. The data will be wrote down and will be analyzed by the researchers at PUCPR. These data will permit the scientists to study the genetic characteristics of dental decay. Upon your authorization, information from your clinical records will be evaluated by the scientists and used in the study.

2) Risk/Side Effects

The physical health risks of participating in this study are very small and limited to the clinical examination. During the examination, you may feel temporary discomfort due to the use of the tongue depressor.

3) Alternatives

If you are affected by dental decay, a visit to a dentist will be indicated, whether or not you decide to participate in the study. Therefore, if you decide not to participate, or to cease your participation in the study at any moment, all procedures for diagnosis as also information about prevention will be given.

If you are not affected by dental decay, you are being asked to participate in the study as part of the relative of an affected individual. In this case, your decision whether or not to participate, or to cease your participation at any moment, will not interfere in any way with medical procedures for diagnosis or treatment of dental decay you may need in the future. Likewise, your decision will not reflect on dental procedures needed by an affected relative or contact you may have.

4) Cost for the participant

In case you decide to participate in the study, there will be no costs for you. All costs will be covered by the study.

5) Benefits

In the long term, the results of this study, to which you will be submitted, may facilitate the detection of dental decay causes, making it possible to avoid inadequate treatments. In addition, it is expected that additional scientific knowledge will be achieved, with consequent improvement of treatment of people affected by dental decay.

6) Reimbursement

You will not be reimbursed for participating in this study

7) Confidentiality of the data

The participation in research projects may result in loss of privacy. In addition, discovery of genetic risk factors for dental decay may expose susceptibilities of certain groups of people, possibly inducing others or certain companies to consider these groups different in a negative way. However, procedures will be adopted by the supervisors of the study in order to protect the confidentiality of the information your dependent provide and the information produced by the project. No individual genetic information will be made public. All information will be coded and kept in a place with restricted access during all times. Only the researchers involved in the study will have access to the information. After the end of the study the information will be transcribed to computer files and will be kept in a place with access restricted to the researchers involved. The data obtained from this study may be discussed with researchers from other institutions, but no identification will be provided.

8) Authorization for the use of the data collected in the study "A study of host genetic risk factors for leprosy susceptibility"

In case you have already been interviewed for the leprosy study, also conducted by our study group and named "A study of host genetic risk factors for leprosy susceptibility", if you sign this informed term you will also authorize us to use the data collected about you and your family in the dental decay study. This authorization will avoid that the researchers make again the same questions, which were asked before.

TERM OF CONSENT

A Genetic Epidemiologic Study of Dental Decay Susceptibility

You will receive and keep a copy of this term of consent. If you have any questions in the future about the participation of your dependent in the study, please don't hesitate to use the following phone numbers and e-mail address to contact the principal investigator of the study:

Prontuary number:_____ File number:_____

Marcelo Távora Mira
Phone number: (41) 3271 2619
Cell phone number: (41) 9164 4045
E-mail address: m.mira@pucpr.br

PARTICIPATION IN THE STUDY IS VOLUNTARY

You have the right not to authorize the participation of your dependent in the study, or even cease his/her participation at any moment you want, with no risk for his/her medical treatment. If you authorize his/her participation, Iyou shall sign or provide your digital print on the line below.

If you authorize the participation of your dependent, you will permit that your address and your phone number be registered on a separate form, in order to facilitate contact when necessary. As discussed before, all the information will be kept confidential.

_____ _____

Full name Prontuary number

_____ _____

Signature of Interviewer Name of Interviewer

_____ _____

Signature of Witness 1 Signature of Witness 2

___/___/___

Date

APPENDIX H: ARTICLE 1

Article accepted for publication at the Journal of Infectious Disease. This research was developed in our group. As part of my PhD training, I conducted the CSA for this research during my stage in Paris, INSERM U550.

A Major Gene Controls Leprosy Susceptibility in a Hyper-Endemic Isolated Population from North of Brazil

FP Lázaro[1,#], RI Werneck[1,#], CCO Mackert[1], FC Prevedello[1], RP Pimentel[1], GMM Macedo[2]; MA Euletério[1], G Vilar[1], L Abel[3,4], MB Xavier[2], A Alcaïs[3,4] and MT Mira[1]*

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ABSTRACT and KEYWORDS

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* that affects 250.000 new individuals worldwide every year. Genetic analysis has been successfully applied to the identification of host genetic factors impacting on susceptibility to leprosy; however, a consensus regarding its mode of inheritance is yet to be achieved. We conducted a Complex Segregation Analysis (CSA) on leprosy using data from the Prata Colony, an isolated, highly endemic former leprosy located at the outskirts of Brazilian Amazon presenting large multiplex, multigenerational leprosy pedigrees. Our enrollment strategy was complete ascertainment leading to the inclusion of the whole colony, totalizing 2005 individuals (225 affected) distributed in 112 pedigrees. CSA was performed using REGRESS, which specified a regression relationship between the probability of being affected and a set of explanatory variables. CSA identified a best fit co-dominant model, with a major gene accounting for the entire familial effect observed. The frequency of predisposing allele was estimated at 0.22. Penetrance for homozygous individuals for the predisposing allele older than 30 years old ranged from 56% to 85%, depending on gender, with higher values for males. Results suggest that the Prata population may be particularly suitable for leprosy gene identification studies.

Keywords: *leprosy; Mycobacterium leprae; complex segregation analysis.*

APPENDIX I: ARTICLE 2

Article accepted for publication at the Journal of Applied Oral Science. In parallel to my PhD study, I provided technical advice and conducted part of the analysis included on this research.

Analysis of the association between lactotransferrin (LTF) gene polymorphism and dental caries

Abstract

Objective. The present study aimed to evaluate the association between lactotransferrin (LTF) gene polymorphism (exon 2, A/G, Lys/Arg) and dental caries. ***Material and Methods****.* A convenient sample of 110 individuals, 12 years old, was divided into: *group 1*, 48 individuals without caries experience (DMFT=0), and *group 2*, 62 subjects with caries experience (DMFT≥1). DNA was obtained from a mouthwash with 3 % glucose solution, followed by a scrapping of the oral mucosa. After DNA purification, polymerase chain reaction (PCR), single strand conformation polymorphism (SSCP) was performed to access the study polymorphism. The LTF A/G (Lys/Arg) polymorphism had been previously reported as located in exon 1. ***Results.*** Allele 1 of the study polymorphism was associated with low DMFT index and showed a protective effect against caries experience (OR=0.16, IC=0.03-0.76, *p*=0.01). ***Conclusion****.* Lactotransferrin A/G (exon 2, Lys/Arg) polymorphism was associated with susceptibility to dental caries in 12-yr-old students.

Key Words: Caries experience. LTF. Gene polymorphism. Exon 2.

APPENDIX J: ARTICLE 3

Article submitted to the Journal of Investigative Dermatology. This research was developed in our group. In parallel to my PhD, I conducted the analysis for this study.

Genetic Variants of the *DDR1* Gene are Associated with Vitiligo in Two Independent Brazilian Population Samples

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Abbreviations: KP, Köebner Phenomenon; SNP, Single Nucleotide Polymorphism; LD, Linkage disequilibrium*; DDR1*, Discoidin Domain Receptor 1.

Abstract

Vitiligo is a chronic disease characterized by macules devoid of melanin and identifiable melanocytes. Adhesion of melanocytes to the basement membrane by integrin CCN3 is mediated through collagen IV receptor DDR1. We hypothesize that genetic variants of the *DDR1* gene are associated with the occurrence of vitiligo. To test this hypothesis, we genotyped 10 *DDR1* tag SNPs in 212 trios composed by an affected child and both parents. Associated markers were then genotyped in 134 independent, unrelated individuals with vitiligo and 134 unrelated controls. Allele “T” of tag SNP rs4618569 was associated with an increased risk for vitiligo in the family trios (P=0.002, OR=5.27; 95% IC=1.59-17.40), whereas allele “C” of tag SNP rs2267641 was associated with an increased risk for vitiligo on both family-based and case-control populations (*P*=0.01, OR=3.47; 95% CI=1.22-9.17; *P*=0.04, OR=6.00; 95% CI=1.73 - 52.33, respectively). Best evidence for association in the trios was obtained for a haplotype composed by risk alleles of markers rs4618569 and rs2267641 (P=0.0006). There was an age-dependent enrichment of rs4618569 “T” allele and rs2267641 “C” allele in early-onset affected individuals. In conclusion, we propose *DDR1* as a new susceptibility gene for vitiligo, possibly implicating a defective cell adhesion in vitiligo pathogenesis.